THE NFκB INDUCING KINASE
-
REGULATING THE FUNCTION OF DENDRITIC CELLS AND γδ T CELLS

Dissertation

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SUMMARY

In order to avoid fatal autoimmunity, the immune system needs to maintain a careful balance between reactivity towards invading pathogens, and tolerance towards self-antigens. One of the most prominent regulators involved in these processes is the transcription factor family of NFκB (nuclear factor kappa-light-chain-enhancer of B cells). Two main pathways can activate NFκB: the classical, and the alternative or non-canonical pathway. In case of the latter the NFκB-inducing kinase (NIK) is the most important signal conveyor.

Signaling via NIK has been shown to be essential for the development of lymph nodes and the normal function of many immune cell types, including B cells, αβ T cells and dendritic cells (DCs). Therefore, mice deficient for NIK show diverse phenotypes in different cellular compartments, which impedes the clarification of the role of NIK in one specific cell type.

Within this thesis, I describe a novel mouse line that allows for conditional deletion of NIK in a time- and tissue-specific manner. Using these $NIK^{flox/flox}$ mice we could show that NIK signaling plays an essential role both in T cells and DCs for the induction of experimental autoimmune encephalomyelitis (EAE), which is the animal model for multiple sclerosis (MS). DC-specific deletion of NIK led to an impaired ability of DCs to produce pro-inflammatory cytokines, and to a selective loss of skin-draining $Langerin^{+}$ DCs in the lymph node. Hence, loss of NIK signaling seems to have very discrete effects on the migration and function of DCs.

Furthermore, we aimed at investigating the development and function of γδ T cells in the absence of functional NIK signaling. γδ T cells are increasingly recognized as important effector cells at the interface of innate and adaptive immunity, particularly due to their ability for immediate secretion of pro-inflammatory cytokines. We found that NIK-deficient lymphoid and tissue-resident γδ T cells were selectively impaired in their ability for IL-17 production, while their expression of IFN-γ remained unchanged. In addition, the pool of dendritic epidermal T cells (DETCs) in the skin of $NIK^{-/-}$ mice was disturbed in that most of the DETCs lost expression of the canonical Vγ5Vδ1 T cell receptor. This was due to a developmental block of $Vγ5^{+}$ precursor cells in the embryonic thymus, most likely caused by the absence of Skint-1 expression on NIK-deficient thymic epithelial cells.

In summary, these results identify a previously unknown role of NIK in the function of γδ T cells. In addition, the novel $NIK^{flox/flox}$ mouse line will be a unique tool for understanding the function of NIK in different immune compartments in more detail.

ZUSAMMENFASSUNG

In einem gesunden Organismus ist die Hauptaufgabe des Immunsystems eindringende Mikroorganismen zu zerstören. Gleichzeitig muss jedoch gewährleistet sein, dass das Immunsystem keine Reaktivität gegenüber körpereigenen Zellen zeigt, da sonst gefährliche Autoimmunerkrankungen die Folge sind. Eines der wichtigsten Moleküle, die dieses Gleichgewicht regeln, ist der Transkriptionsfaktor NFκB. NFκB bezeichnet eine Familie an genregulatorischen Proteinen, die über zwei verschiedene Signalwege aktiviert werden, nämlich über den sogenannten klassischen und den alternativen Weg. Für die alternative Aktivierung von NFκB ist die sogenannte NFκB inducing kinase (NIK) das wichtigste Signalprotein.

Bisher ist bekannt, dass NIK essentiell für die Entstehung von Lymphknoten ist, aber auch für die Funktion von B Zellen, αβ T Zellen und dendritischen Zellen (DCs). Auf Grund dieser vielfältigen Funktionen war es bisher schwierig, die Rolle von NIK in einzelnen Zelltypen zu untersuchen.

Im Rahmen dieser Arbeit beschreibe ich einen neuen Mausstamm, der ein zelltyp-spezifisches Ausschalten des Gens für NIK erlaubt ($NIK^{flox/flox}$). Mit Hilfe dieser $NIK^{flox/flox}$ Tiere konnten wir zeigen, dass Signale via NIK essentiell für die Entstehung von experimenteller autoimmuner Enzephalomyelitis (EAE) sind, die üblicherweise als Tiermodell für Multiple Sklerose (MS) angesehen wird. Ein spezifisches Ausschalten von NIK in DCs führte dazu, dass diese weniger proinflammatorische Zytokine produzieren konnten. Außerdem war die Anzahl an $Langerin^{+}$ DCs im hautnahen Lymphknoten drastisch reduziert. Daher schlussfolgern wir, dass NIK eine wichtige Rolle für die Migration und Funktion von DCs hat.

Außerdem wollten wir die Funktion von NIK in einer Zellpopulation prüfen, die in diesem Kontext noch nicht eingehend untersucht wurde, nämlich γδ T Zellen. Innerhalb der letzten Jahre wurde klar, dass γδ T Zellen wichtige Effektorzellen an der Schnittstelle zwischen adaptivem und angeborenem Immunsystem sind, vor allem wegen ihrer Fähigkeit schnell pro-inflammatorische Zytokine produzieren zu können. Wir konnten zeigen, dass sowohl lymphoide als auch gewebe-ansässige γδ T Zellen kein Interleukin-17 mehr produzieren konnten, während ihre Fähigkeit zur Produktion von Interferon-γ unbeeinträchtigt war. Außerdem zeigten $V\gamma5^{+}$ dendritische epidermale T Zellen (DETCs) Unregelmäßigkeiten, die wir auf eine Blockade in der Entwicklung von $V\gamma5^{+}$ Vorläuferzellen zurückführen konnten.

Kurz zusammengefasst zeigen diese Experimente, dass NIK eine bisher unbekannte Rolle in der Funktion von γδ T Zellen hat. Vor allem aber wird das konditionale Mausmodell ($NIK^{flox/flox}$) ein nützliches Werkzeug sein, um die Funktion von NIK innerhalb des Immunsystems genauer zu untersuchen.

ABBREVIATIONS

Ab	antibody
Ag	antigen
APC	antigen presenting cell
approx.	approximately
BBB	blood brain barrier
β-ME	β-mercaptoethanol
BM	bone marrow
BMC	bone marrow chimera
bp	base pair
BSA	bovine serum albumin
°C	temperature in degrees Celsius
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CFA	Complete Freund's Adjuvant
CFSE	carboxyfluorescein succinimidyl ester
cDNA	complementary DNA
CNS	central nervous system
Cre	LoxP site-specific recombinase
CTL	cytotoxic T lymphocyte
d	day/s
DC	dendritic cell
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene-diaminetetraacetic acid
ELISA	enzyme-linked immuno-sorbent assay
ES cell	embryonic stem cell
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	Figure
Flp	FRT site-specific recombinase
FoxP3	forkhead box protein 3
FRT	Flp recombination target
g	gram
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage-colony stimulating factor
h	hour/s
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HLA	human leukocyte antigen
i.p.	intraperitoneally
i.v.	intravenously
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin

IRES	internal ribosome entry site
LN	lymph node/s
loxP	recognition sequence for Cre
LPS	lipopolysaccharide
M	molar
MACS	magnetic activated cell sorter
MBP	myelin basic protein
MFI	mean fluorescence intensity
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MS	multiple sclerosis
MΦ	macrophage/s
n	nano
NaCl	sodium chloride
NaOH	sodium hydroxide
Neo(R)	neomycin resistance gene
o/n	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PP	Peyers patch
Ptx	Pertussis toxin
RAG	recombination activating gene
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
sec	seconds
s.c.	subcutaneously
SD	standard deviation
SEM	standard error of mean
SN	supernatant
Taq	polymerase from Thermus aquaticus
TCR	T cell receptor
TE	Tris-EDTA buffer
TEC	thymic epithelial cell
tg	transgenic
TGFβ	transforming growth factor-β
T_H cell	helper T cell
TLR	toll-like receptor
Tregs	regulatory T cells
µl	microliter
µM	micromolar
3′	three prime end of DNA sequences
5′	five prime end of DNA sequences

INTRODUCTION

THE IMMUNE SYSTEM

The term "immune system" covers all molecular and cellular mechanisms that an organism uses to defend itself against invading pathogens and resulting disease. For higher vertebrates, the immune system consists of a complex network of specialized cells and organs, but even simple unicellular organisms such as bacteria use enzymes to protect themselves for example from bacteriophage infections.

In the case of all jawed vertebrates, the immune system can be divided into two mechanistically distinct units, namely innate and adaptive immunity. The innate immune system primarily relies on pattern recognition receptors (PRRs), which recognize evolutionary conserved structures on pathogenic organisms, so called pathogen associated molecular patterns (PAMPs). Beside that, innate immune cells are also able to sense stress signals sent out from injured or damaged cells.

Hence, the innate immune system can react immediately to a wide range of pathogens, but neither offers specificity (beyond the recognition of PAMPs) nor immunological memory. As a result, vertebrates have evolved the adaptive immune system, which is not only able to recognize any invading organism in a highly specific manner, but also offers immunological memory. For the purpose of this thesis, we will focus on the function of the immune system in mammals, with a main emphasis on the murine immune system as a representative model for human immune responses.

THE INNATE IMMUNE SYSTEM

Innate immunity represents the evolutionary older arm of the immune system, which provides immediate defense against a wide range of invading pathogens in a non-specific way.

In simplified terms, the innate immune system can be divided into three parts: physical barriers, the complement system and innate immune cells. Epithelial surfaces provide the first line of defense against any intruder, and if not injured, the lung, gut and skin epithelium represent very tight and impermeable physical barriers. While the purpose of epithelia obviously extends beyond their role as immunological barriers, the complement system evolved solely as a primitive defense system. It consists of a variety of plasma proteins, which can either lead directly to the destruction (lysis) of pathogens, or rather recruit additional immune cells (reviewed in (Fujita, 2002)).

Prototypic innate immune cells are natural killer cells (NK cells), mast cells, eosinophils, basophils and neutrophils. Dendritic cells (DCs) and macrophages are phagocytic cells of the innate immune system that are able to engulf and destroy pathogens, but these two cell types fulfill additional roles in activating adaptive immunity, as will be discussed further below.

THE ADAPTIVE IMMUNE SYSTEM

Compared to innate immunity, adaptive immunity has two additional key features: First, the ability to recognize almost any possible microbial structure in a highly specific way, and second, the ability to memorize prior encounters for mounting a faster and stronger secondary response. This property has been termed immunological memory (Ahmed and Gray, 1996).

The cells of the adaptive immune system are T and B lymphocytes. The latter are generated in the bone marrow and are responsible for the production of antibodies, thereby being major players in the so-called humoral immune response. In contrast, T cells are generated in the thymus and are the main players in cellular immune responses. The principal functions of T cells are the activation and regulation of other immune cells by the secretion of soluble messenger molecules termed cytokines, and the lysis of virus infected cells.

The exceptional feature of T and B cells is their expression of a unique antigen receptor, namely the T cell receptor (TCR) and the B cell receptor (BCR). However, there is a major difference in the mode of antigen recognition: T cells are recognizing peptide antigens, which need to be presented by antigen presenting cells such as dendritic cells (DCs) in the context of the major histocompatibility complex (MHC) after processing of the protein (van der Merwe and Davis, 2003). In contrast, B cells are able to recognize soluble antigens in their naïve form without the need for further processing.

So how can adaptive immune cells be able to recognize virtually any antigen without prior encounter? The key mechanism underlying this phenomenon is the random somatic gene rearrangement of a number of DNA fragments (the variable, diversity and joining loci, VDJ) during lymphocyte development (Fugmann et al., 2000). It is estimated that together with additional means to increase receptor diversity, namely junctional diversity during joining of the V, D and J loci, up to 10^{11} to 10^{15} different amino acid sequences for the BCR and TCR, respectively, can be generated.

Due to the random nature of this process, not all of the resulting amino acid sequences will provide useful receptors. On top of that, some of these receptors will be recognizing self-antigen, i.e. molecules present in the organism itself. As a result,

the repertoire of developing adaptive immune cells needs to be selected carefully, which will be discussed in more detail in subsequent chapters.

LYMPHOID TISSUES

From an evolutionary perspective, the advent of adaptive immune cells concurred with the appearance of lymphoid tissues (Hofmann et al., 2010). In higher vertebrates, lymphoid tissues are not only the place where immune cells develop, but more importantly provide a specialized microenvironment that allows the efficient interaction between innate and adaptive immune cells. Three types of lymphoid tissues can be distinguished: primary lymphoid tissues (PLTs) are the bone marrow (BM) and the thymus, secondary lymphoid tissues (SLTs) include lymph nodes (LNs), the spleen and Peyer´s Patches (PPs), and tertiary lymphoid tissues are simple lymphoid structures that can develop in situations of chronic inflammation (Aloisi and Pujol-Borrell, 2006).

The bone marrow is the principal site where hematopoietic stem cells (HSCs) reside, which will give rise to all cells of the hematopoietic lineage including erythrocytes, myeloid and lymphoid cells (reviewed in (Cumano and Godin, 2007)). B cells derive from a lymphoid progenitor, and will continue to develop in the bone marrow. In contrast, T cells, which also derive from a common lymphoid progenitor in the bone marrow, will mature in the thymus. The process of T cell development will be discussed in more detail in the following chapters.

Different to the bone marrow and the thymus, lymph nodes and spleen provide specialized anatomical structures to favor the initiation of adaptive immune responses by bringing innate and adaptive immune cells into close proximity (Junt et al., 2008). These two main SLTs differ in their precise function: the spleen primarily samples blood-borne antigens, while lymph nodes are positioned strategically to collect the draining fluid from certain peripheral tissues. Peyer´s patches in turn are comparably smaller lymphoid structures that are not connected to the lymphoid drainage system and are only found in the gut, where they sample antigen from the gut lumen.

Despite their different functions, the basic building blocks of spleen and lymph node are comparable: an outer "antigen-sampling" zone containing large numbers of antigen-presenting cells (mostly DCs), and distinct inner T and B cell zones (Junt et al., 2008). During homeostasis, T cells will circulate throughout all lymphoid tissues and scan antigen-presenting cells for their cognate antigen. Upon recognition of their target, they will get activated and initiate an adaptive immune response.

INITIATING AN ADAPTIVE IMMUNE RESPONSE

Usually, the purpose of an immune response is to combat and ultimately eliminate an intruding pathogen, often a microbe or a virus.

In a nutshell, there are three steps during an adaptive immune response: first, the invading pathogen will encounter tissue-resident innate immune cells, among them DCs. These sense the invader via pattern recognition receptors, followed by activation, and phagocytosis and processing of foreign antigens. Activated DCs migrate to the draining lymph node and upregulate expression of MHC class II and costimulatory molecules. Following the arrival in the draining LN, circulating T cells get into contact with DCs, and after recognition of their cognate antigen via the TCR become activated. It is thought that full T cell activation requires three signals: the antigen, costimulation via the CD28-CD80/86 axis and polarization via cytokines (Gutcher and Becher, 2007). This process is usually referred to as the priming phase of an immune response, and lack of costimulatory signals can result in T cell anergy, i.e. unresponsiveness towards the antigen.

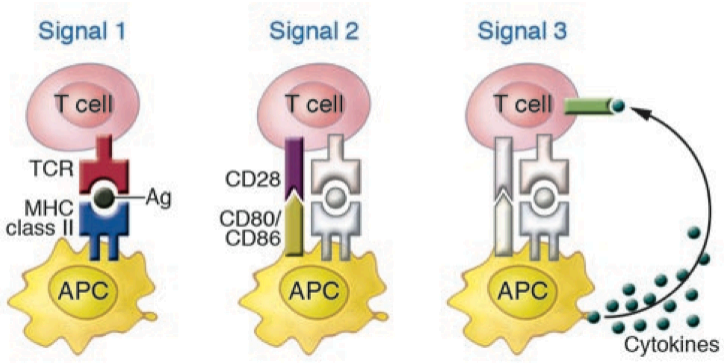

Figure 1: Three signal-hypothesis for the activation of T cells.

After formation of the immunological synapse between antigen-presenting cell (APC) and T cell, three signals are needed for full T cell activation and differentiation: first, recognition of the cognate peptide antigen presented by MHC class I or II molecules. Second, costimulation via membrane bound surface receptors of the B7-family, primarily CD80 and CD86. Third, polarization signals via cytokines will dictate the differentiation of activated T cells towards a particular effector lineage.
Ag: antigen. TCR: T cell receptor. MHC: major histocompatibility complex.
(picture modified from Gutcher et al, JCI 2007)

The second phase of an adaptive immune response is the so-called effector phase: antigen-specific T cells and B cells will undergo clonal expansion, which is one of the reasons why it takes several days until adaptive immunity reaches its full efficiency. During their activation, T cells will also undergo functional differentiation towards a certain effector program, leave the lymph node and migrate to the site of

inflammation. There, the invading pathogen will be combated by a variety of effector mechanisms, usually involving both adaptive and innate immune cells. In fact, one of the main roles of adaptive immunity is to enhance and steer the effector functions of innate immunity.

The third phase of an immune response is the return to homeostasis and formation of memory. Most of the expanded antigen-specific T and B cells will die by apoptosis, while some of them will become memory cells, that persist in the bone marrow or the periphery and during secondary stimulation can expand and react very rapidly (reviewed in (Mueller et al., 2013) and (McHeyzer-Williams and McHeyzer-Williams, 2005)).

Within the scope of this thesis we will focus mainly on DCs, and T cells and their effector mechanisms, which include secretion of cytokines for activation of other immune cells, lysis of target cells, and also regulation of immune responses.

THE OTHER SIDE OF THE COIN: AUTOIMMUNITY

As already mentioned, the receptor repertoire of adaptive immune cells is generated by a largely random process that inevitably will generate receptors capable of recognizing self-antigen, i.e. constituents of the organism itself. Several mechanisms have evolved to remove this potentially dangerous specificities from the T cell and B cell repertoire during development, some of which we will discuss in further detail below (Boehmer and Melchers, 2010). Also, so-called dominant tolerance mechanisms exist, such as immunosuppressive immune cell subsets and the induction of anergy, which is the unresponsiveness of adaptive immune cells towards certain antigens (Fathman and Lineberry, 2007).

However, if any of these machineries fails, attack of self-antigen by the immune system will lead to autoimmune disease, which has already been proposed by the visionary Paul Ehrlich at the beginning of the 20th century, back then termed "horror autotoxicus". By now it is known that the most prominent autoimmune diseases include diabetes mellitus type 1, systemic lupus erythematosus (SLE), rheumatoid arthritis, Grave´s disease and multiple sclerosis (MS) (reviewed in (Steinman, 1995). In Western civilisations, up to 5% of the population suffer from autoimmune pathologies.

Hence, it is indispensable to understand the molecular mechanisms that enable and drive autoimmunity in order to develop treatment strategies. Within this thesis we will discuss only one the above-mentioned diseases, namely MS and its animal model experimental autoimmune encephalomyelitis (EAE). Before we do so, we will explain the function and development of T cells, and the mechanisms that ensure immunological tolerance in a healthy individual.

αβ T CELLS

It was only in the 1960s that bulk lymphocytes were separated into B and T cells based on their functionally distinct roles in humoral and cellular immunity (Miller and Mitchell, 1967). Roughly 10 years later the first sub-classification of T cells based on their differential expression of Ly antigens was proposed (Cantor and Boyse, 1975; Kisielow et al., 1975). By now it is well established that two main classes of αβ T cells exist, which can be distinguished based on the surface expression of different CD (cluster of differentiation) molecules: $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells (T_H cells).

$CD8^+$ T cells recognize peptide antigens in the context of MHC class I molecules and are able to directly lyse target cells via cytotoxic granules, which is a critical effector mechanism for the clearance of virus-infected cells (Wong and Pamer, 2003). In contrast, $CD4^+$ T cells recognize peptide antigens bound to MHC class II. Their main role is the secretion of soluble messenger molecules termed cytokines that shape the subsequent immune response, and the provision of help to B cells and $CD8^+$ T cells (Zhu et al., 2010). Prior to the encounter with their cognate antigen, $CD4^+$ T cells are referred to as "naïve", and antigen recognition in combination with additional signals will trigger not only their activation, but also their differentiation towards a certain effector profile.

A seminal step forward in the functional understanding of $CD4^+$ T cells was achieved in 1986, when Coffman and colleagues characterized different $CD4^+$ T cell clones based on their expression of the effector cytokines Interferon-γ (IFN-γ) and Interleukin-4 (at this time known as B cell stimulating factor 1). IFN-γ producing T cells were termed T_H1 cells, and IL-4 producing T cells became known as T_H2 cells (Mosmann et al., 1986). This early dogma of helper T cell differentiation became widely accepted, but in the past decade several additional sub-classes of $CD4^+$ T cells have been identified, including immunosuppressive regulatory T cells. The phenotypic properties as well as the effector mechanism of these subsets will be discussed in more detail in the next section.

αβ T CELL EFFECTOR LINEAGES AND SIGNATURE CYTOKINES

The past decade has witnessed the discovery of many additional subsets of $CD4^+$ T helper cells in addition to the classical T_H1 and T_H2 lineages, namely T_H17 cells, follicular helper T cells (T_{FH}) and natural and inducible regulatory T cells (Tregs). However, whether these T cell lineages (which have been mostly based on the signature cytokines produced) really represent a terminal differentiation state *in vivo*, or whether there is functional plasticity depending on the current microenvironment is a matter of intense debate (Zhou et al., 2009).

The differentiation towards the T_H1 lineage is thought to be induced by IL-12, which is produced by activated DCs. Naïve T cells sensing IL-12 (in addition to TCR stimulation) will activate a signaling cascade via the signal transducer and activator of transcription 4 (STAT-4) (Kaplan et al., 1996), leading to activation of the T box transcription factor T-bet, which is considered to be the master regulator of T_H1 cells (Szabo et al., 2000). Functionally, T_H1 cells aid the eradication of intracellular bacteria by production of IFN-γ, but also IL-2 and Lymphotoxin-α (LTα). IFN-γ, the signature cytokine of T_H1 cells, acts as a major activator of macrophage phagocytosis.

In contrast, T_H2 differentiation requires signaling via IL-4 and STAT-6 (Kopf et al., 1993), leading to activation of the transcription factor GATA-3 (Zheng and Flavell, 1997). The signature cytokines of T_H2 cells are IL-4, IL-5 and IL-13, and the main function of T_H2 cells is the induction of humoral (i.e. antibody mediated) immunity, thereby leading to the containment of extracellular pathogens. Also, production of IL-5 by T_H2 cells will activate eosinophils to attack parasites like helminthes.

Notably, for both T cell lineages the secreted signature cytokines will reinforce their given differentiation state: IFN-γ and IL-2 further activate T-bet expression in T_H1 cells, while IL-4 reinforces GATA-3 expression and suppresses T_H1 development by blocking IL-12 receptor expression (Ouyang et al., 2000; Szabo et al., 1997).

The T_H1-T_H2 dogma dominated research on the function of T helper cells for over 20 years, until it was shown that stimulation of naïve T cells in the presence of IL-23 leads to secretion of IL-17, a cytokine which is produced neither by T_H1 nor T_H2 cells (Aggarwal et al., 2003). In 2005, the term T_H17 cell was first used to describe these T cells, which led to the dawn of a third T helper cell lineage (Harrington et al., 2005; Langrish, 2005; Park et al., 2005). Shortly later, it was revealed that the orphan nuclear receptor RORγt is necessary for the generation of IL-17 producing T cells (Ivanov et al., 2006). However, the receptor for IL-23 (IL23-R) is not expressed on naïve T cells, but only upregulated after stimulation in the presence of IL-6 (McGeachy et al., 2007; Zhou et al., 2007).

The main reason why T_H17 cells attracted a huge interest among immunologists is the fact that this subpopulation of T cells has been implicated in various models of autoimmunity, for most of which IL-23 was shown to be a non-redundant player (Croxford et al., 2012; Cua et al., 2003; Langrish, 2005). We will discuss the role of pathogenic T cells in autoimmunity in subsequent chapters.

Roughly at the same time as IL-23, the forkhead/winged helix transcription factor FoxP3 was discovered as being a master regulator for the development of regulatory T cells (Tregs) (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). While it has already been known for a while that $CD25^{hi}$ $CD4^{+}$ T cells have

immunosuppressive capacity (Sakaguchi et al., 1995), the discovery of FoxP3 firmly established the existence of a regulatory T cell population. In the mouse model, both complete deletion of FoxP3 as well as depletion of $FoxP3^+$ Tregs causes fatal autoimmunity (Kim et al., 2007). Notably, while the major fraction of $FoxP3^+$ Tregs is thought to arise in the thymus (nTregs), naïve T cells can be converted to induced Tregs (iTregs) *in vitro* by stimulation with TGF-β (Chen et al., 2003). Seminal experiments utilizing *in vivo* stimulation with low doses of antigen in the absence of costimulation further supported the notion that Tregs can be induced peripherally (Kretschmer et al., 2005).

T follicular helper (T_{FH}) cells differ from the subsets mentioned above in that they do not participate in the effector phase of an immune response directly, but instead promote the so-called affinity maturation of B cells during the later stage of an adaptive immune response. Affinity maturation of B cells occurs in the germinal centers (GCs) of lymph nodes and relies on a process called somatic hypermutation (Odegard and Schatz, 2006). T_{FH} cells are characterized by expression of the chemokine receptor 5 (CXCR-5) and depend on IL-21 and STAT-6 signaling for their differentiation, but as for other T cell subsets it is not yet clear whether these cells represent a distinct lineage (Nurieva et al., 2008).

γδ T CELLS

Both $CD4^+$ and $CD8^+$ T cells express a dimeric TCR that consists of a clonally variable α and β chain. After a long and elusive search by many immunologists, these chains were identified and cloned in the mid 1980s (Hedrick et al., 1984; Saito et al., 1984). Shortly after that, it was reported that there are T cells expressing alternative TCR chains, namely the γ and δ chain (Garman et al., 1986; Heilig and Tonegawa, 1986). In subsequent years it became evident that γδ T cells represent a separate T cell lineage distinct from αβ T cells, with a peculiar enrichment in epithelial tissues (Allison and Havran, 1991). Nevertheless, the immunological community mainly ignored γδ T cells, most likely because of their low abundance in lymphoid organs and the lack of appropriate tools to study their function (Hayday, 2000). Only in the past decade it became obvious, that γδ T cell have unique features that place them at the interface of the adaptive and the innate immune system (Vantourout and Hayday, 2013). This notion was further corroborated by the recent finding that in lampreys, which are primitive jawless vertebrates, a lymphocyte lineage exists that resembles mammalian γδ T cells in terms of development and function (Hirano et al., 2013). Hence, already in the last common vertebrate ancestor roughly 500 million years ago, evolution has shaped three

primordial lymphocytes lineages (B cells, αβ T cells and γδ T cells) with comparable functional properties that have been conserved since then.

The most notable difference between αβ T cells and γδ T cells is that the latter are not restricted to recognizing peptide-MHC complexes. Several qualitatively different antigens have been proposed, including phospho-antigens (Constant et al., 1994), MHC-related proteins including so-called stress antigens (Crowley et al., 1997; Shin et al., 2005) and lipids. Very recently, it has been reported that both human and murine γδ T cells can bind to Phycoerythrin, an algal fluorescent protein, which is also recognized by B cells (Zeng et al., 2012). However, the full scope of antigens that can be recognized by γδ T cells is still a matter of intense research (Vantourout and Hayday, 2013).

Another extraordinary difference to αβ T cells is that many γδ T cell populations show a restricted TCR diversity. This is particularly striking because the combinatorial diversity of the γδ gene locus would in theory be even larger than the one of the αβ locus, namely 10^{20} receptor specificities (compared to theoretical 10^{15} receptors for αβ T cells) (Carding and Egan, 2002). Hence, some mechanism must ensure that the γδ T cell repertoire is focused on few specificities, and it is still unclear how this is achieved (Vantourout and Hayday, 2013).

In addition, γδ T cells of a given TCR specificity often directly home to particular tissues with only minor recirculation through the lymphatic system. One prime example of this phenomenon are so-called dendritic epidermal γδ T cells (DETCs), which are found only in the murine epidermis and show a nearly monoclonal expression of a $V\gamma5^+V\delta1^+$ TCR (Asarnow et al., 1988). Another example are $V\gamma6^+$ γδ T cells, that home primarily to the lung and the reproductive tract, and $V\gamma7^+$ γδ T cells that populate the intestine (Vantourout and Hayday, 2013). Also in the human system, γδ T cell populations of limited diversity have been described, such as the $V\gamma9^+V\delta2^+$ pool in the peripheral blood (Triebel et al., 1988).

As will be discussed in subsequent chapters, γδ T cells differ significantly from αβ T cells in that their ability to produce certain pro-inflammatory cytokines is already pre-programmed during thymic development. This concept has been put forward by several reports (Jensen et al., 2008; Ribot et al., 2009), and was recently corroborated by an extensive transcriptome analysis of various γδ thymocytes subsets (Narayan et al., 2012), which we will discuss in further detail in the chapter on γδ T cell development.

In the next section we will examine some of the effector functions of γδ T cells, with a particular focus on lymphoid stress-surveillance.

γδ T CELL EFFECTOR FUNCTIONS

Similar to αβ T cells, both human and mouse γδ T cells after activation can secrete various effector cytokines, including IFN-γ, TNFα, IL-17, but also IL-4 or granulocyte-macrophage colony stimulating factor (GM-CSF). Obviously, this allows γδ T cells to contribute significantly to an immune response (for example by IL-17 mediated recruitement of neutrophils), but they do so with different kinetics and different efficacy.

As mentioned above, several γδ T cell populations express a very narrow or even monoclonal TCR repertoire, which means that despite being outnumbered by αβ T cells as a lineage, the absolute number of γδ T cells recognizing a particular antigen is relatively large. Hence, after stimulation via the TCR or via certain cytokines, γδ T cells can rapidly mobilize their effector function without the need for clonal expansion (Vantourout and Hayday, 2013).

Based on this fast responsiveness and their ability to recognize stress antigens (such as the MHC class 1b protein Rae-1), the term "lymphoid stress-surveillance" has been termed (Hayday, 2009). This refers to the ability of γδ T cells to rapidly react towards non-microbial stress such as physical perturbations of epithelial cell layers or malignant cell transformation. Supporting this hypothesis, TCRδ$^{-/-}$ animals show increased susceptibility to carcinogenesis in the skin (Girardi et al., 2001), and in certain genetic backgrounds such as FVB or NOD (non-obese diabetic), TCRδ$^{-/-}$ animals develop spontaneous dermatitis (Girardi et al., 2002). Also in humans, the stress-induced MHC like molecules MICA and MICB were shown to stimulate intestinal Vδ1^{+} γδ T cells (Groh et al., 1996; 1998).

The link between genotoxic stress and immune responses was emphasized by another report in which the DNA damage machinery directly induced expression of Rae1 (Gasser et al., 2005). Rae1 is a ligand for the NKG2D (natural killer group 2, member D) receptor being expressed by NK-cells, but also by γδ T cells. Furthermore, in a genetic mouse model in which Rae-1b expression was induced *de novo* in otherwise healthy skin, both Langerhans cells and dendritic epidermal γδ T cells (DETCs) became activated (Strid et al., 2008). Furthermore, DETCs have also been implicated in wound healing (Jameson et al., 2002).

An interesting feature of γδ T cells is that a large proportion constitutively expresses the receptor for IL-23 (Awasthi et al., 2009). Thus, in contrast to αβ T cells that require prior stimulation, γδ T cells can sense IL-23 immediately and respond by production of IL-17 (Sutton et al., 2009), which in turn will induce the recruitment of neutrophils to the site of inflammation. There is increasing evidence that this early inflammatory response induced by tissue-resident γδ T cells is important for optimal control of bacterial infections, as has been shown both in mice and men (Peng et al.,

2008; Umemura et al., 2007). During infection with *Mycobacterium tuberculosis*, γδ T cells even exceed αβ T cells in terms of production of IL-17 (Lockhart et al., 2006). Along these lines, γδ T cells were shown to express the Toll like receptors TLR1 and TLR2 and also sense bacterial products *in vivo* (Martin et al., 2009). In addition to that, it has been reported that IL-23-induced activation of γδ T cells can participate in the pathogenesis of autoimmune models such as psoriatic skin inflammation and experimental autoimmune encephalomyelitis (EAE) (Cai et al., 2011; Pantelyushin et al., 2012; Petermann et al., 2010).
In summary, the importance of different γδ T cell subsets both during protective immune reactions as well as pathologic autoimmune conditions is becoming increasingly evident, emphasizing the need to understand the molecular pathways regulating their development and function in more detail.

So far we have discussed the basic components of innate and adaptive immunity, focusing on different subsets of T cells and their involvement during immune responses. In the next chapters we will have a detailed look at T cell development of the αβ and γδ lineage, as well as the mechanisms of central tolerance.

T CELL DEVELOPMENT

While B cell development occurs in the bone marrow, T cell development progresses in the thymus, a two-lobed organ situated between the heart and the sternum, which can be divided into a cortical and a medullary region and is surrounded by a capsule. Historically, the ancient Greek considered the thymus as the seat of the soul, and it was only in the early 1960s that Jacques Miller could show by neonatal thymectomy in mice that the thymus is essential for the generation of mature T cells (Miller, 1961).
Of note, the thymus does not contain any hematopoietic self-renewing stem cells, but is dependent on the import of lymphoid progenitor cells from the bone marrow (Foss et al., 2001). These cells enter the thymus at the cortico-medullary junction, and within a period of 2-3 weeks have to go through different thymic compartments and a complex series of selection events, leaving the thymus as mature $CD4^+$ or $CD8^+$ αβ T cells, or mature γδ T cells. Notably, T cell development strongly depends on signals derived from different stromal cells present in the thymus, namely cortical and medullary thymic epithelial cells (cTECs and mTECS, respectively) and thymic dendritic cells (DCs) (Anderson et al., 2007).

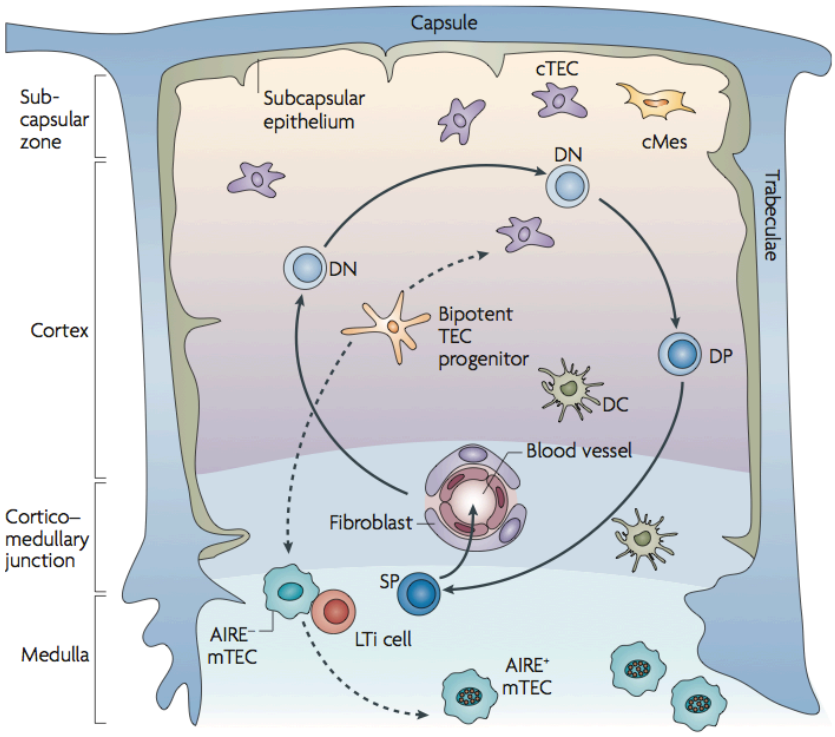

Figure 2: Overview of T cell development and the stromal cell types present in the thymus.

As discussed in the main text, T cell precursors will enter the thymus at the cortico-medullary junction. Double negative (DN) cells migrate towards the capsule and differentiate to double positive (DP) αβ thymocytes, or to γδ thymocytes. $CD4^+$ $CD8^+$ DP thymocytes will be positively selected, and mature to either $CD4^+$ or $CD8^+$ single positive thymocytes. These will migrate into the medulla, where they will interact with medullary thymic epithelial cells (mTECs) that express tissue-restricted self-antigens under control of the autoimmune regulator (Aire). Aire expression in mTECs might be induced by LTIs as well as by early γδ T cell precursors. LTi: lymphoid tissue inducer cell. cTEC: cortical thymic epithelial cell. cMes: cortical mesenchymal cell. DC: dendritic cell
Picture modified from Anderson et al, Nat Rev Immunol 2007.

As they enter the thymus, multipotent lymphoid progenitors (MLPs) are not committed to the T cell lineage yet (Porritt et al., 2004). These early thymocytes are usually termed double negative (DN) cells due to their lack of CD4 and CD8 expression, and their T cell receptor genes are still in germline configuration. Based on expression of CD25 and CD44 four different developmental stages are typically distinguished: DN1 ($CD25^-$ $CD44^+$), DN2 ($CD25^+$ $CD44^+$), DN3 ($CD25^+$ $CD44^{low}$), DN4 ($CD25^-$ $CD44^{low}$). Within these thymocyte compartments, further subsets with defined developmental potential can be identified by the use of additional surface markers such as c-kit (CD117) and heat stable antigen (CD24) (reviewed in (Petrie and Zúñiga-Pflücker, 2007)).

After extensive proliferation at the DN1 stage, developing thymocytes migrate from the cortico-medullary junction towards the capsule and progress to the DN2 and DN3 stage, where final commitment to the T cell lineage is taking place. At these stages, expression of the RAG (recombination activating genes) enzymes is induced, leading to the rearrangement of the T cell receptor genes. Notable, the early DN3 stage is also the bifurcation point at which αβ and γδ T cell development diverges.

αβ T CELL DEVELOPMENT

For αβ T cells, at the DN3 state rearrangement of the β gene locus is occurring. The β locus harbors a number of so-called V-, D- and J-gene segments, which will be rearranged by RAG-1 and RAG-2 to yield a continuous in-frame VDJ sequence coding for the TCR β chain. This β chain will pair with an invariant pre-Tα chain as well as CD3 molecules to form the pre-T cell receptor, which seems to be signaling in a ligand-independent fashion (Irving et al., 1998). Signaling via the pre-TCR is essential for developing αβ thymocytes, and all cells that fail to successfully express their β chain will die by apoptosis (Petrie and Zúñiga-Pflücker, 2007). This process is usually referred to as β-selection, representing the first developmental checkpoint that ensures the functionality of newly formed αβ T cells.

Following expression of the pre-TCR, a series of profound changes is induced, marking the progression to the DN4 stage: VDJ rearrangement at the second allele is suspended (allelic exclusion), CD4 and CD8 mRNA is expressed, and cells increase their proliferative activity massively (Petrie and Zúñiga-Pflücker, 2007). Concomitant, rearrangement of the TCR α locus is started, which comprises only V and J segments. Functional α chains will replace the pre-T α chain and together with the β chain form a mature T cell receptor. Simultaneous with the mature TCR, CD4 and CD8 protein will be expressed on the surface, marking the developmental transition from the DN to the double positive (DP) stage (Petrie et al., 1990).

DP thymocytes reside in the subcapsular zone of the thymus where they extensively interact with cortical thymic epithelial cells (cTECs) in order to pass the next developmental checkpoint, namely positive selection. At this stage, only DP cells binding with a low avidity to self-antigen in the context of self MHC complexes will receive a survival signal, while failure to do so will induce apoptosis. Hence, positive selection ensures restriction of the mature T cell repertoire to self MHC molecules (Zinkernagel et al., 1978).

Only 5-10% of developing DP thymocytes will survive the positive selection checkpoint and will start migrating back to the thymic medulla. Notably, the efficiency of this process is higher than random TCR gene rearrangement would

suggest, and it was reported recently that germline encoded residues in the αβ TCR facilitate recognition of MHC molecules (Scott-Browne et al., 2009).

During their migration to the medulla, DP thymocytes continue to develop either to CD4 or CD8 single positive (SP) αβ T cells (reviewed in (Starr et al., 2003)). Obviously this lineage decision depends on the specificity of the TCR: $CD4^+$ T cells only recognize MHC class II molecules, while $CD8^+$ T cells only recognize MHC class I molecules. The mechanism underlying CD4/CD8 lineage differentiation is still incompletely understood, and different models have been proposed (Singer et al., 2008): first, a stochastic model, in which CD4 and CD8 co-receptors are randomly expressed, with only those cells surviving that will still bind MHC molecules. Second, an instructive model, in which MHC I restricted TCRs actively repress CD4 expression and vice versa (Germain, 2002). However, in the past decade several lines of evidence have pointed towards a more complex model, which has been termed "coreceptor reversal": DP thymocytes that have undergone positive selection will downregulate CD8 by default, concomitant with upregulation of IL7Rα expression. If the ensuing loss of CD8 signaling does not disrupt the TCR signaling, cells will progress towards $CD4^+$ T cells. In contrast, if the loss of CD8 interferes with TCR signaling, IL7Rα signaling (which is otherwise antagonized by the TCR) will lead to silencing of CD4 expression and reintroduce CD8 expression (Bosselut et al., 2003; Brugnera et al., 2000; Yu et al., 2006).

Both CD4 and CD8 single positive thymocytes will migrate further inwards into the thymus, leaving the cortical zone and entering the thymic medulla. This migration step is dependent on chemokine signaling via the chemokine receptor 7 (CCR7), and ensures the next critical selection step during T cell development, which is the tolerization of the T cell repertoire to self-antigens (Kurobe et al., 2006). The corresponding ligands for CCR7, CCL19 and CCL21 are expressed by medullary thymic epithelial cells (mTECs) (Misslitz et al., 2004).

As mentioned above, the random nature of TCR gene rearrangement generates a vast diversity of TCR specificities, which inevitably will also include some self-antigens. Hence, to avoid potentially fatal destruction of the body's own organs, self-reactive T cells need to be purged from the repertoire. Furthermore, a fraction of developing $CD4^+$ thymocytes will be diverted towards $FoxP3^+$ regulatory T cells (Tregs). These processes are mediated by interactions with mTECs and thymic DCs, and will be further discussed in the next chapter in the context of central tolerance.

Interestingly, recent evidence suggests that not all αβ T cells leave the thymus as naïve cells, but that a small fraction of $CD4^+$ T cells is pre-committed towards IL-17 production (Marks et al., 2009). These cells show preferential usage of certain Vβ-chains, and similar to $FoxP3^+$ Tregs depend on self-antigen presentation by mTECs

for their development (Kim et al., 2011; Marks et al., 2009). However, the specificity as well as the precise function of these cells remains unknown, but it is important to note that the thymus can be able to select T cells with a pre-defined function (Stritesky et al., 2012).

CENTRAL TOLERANCE

Subsequent to the CD4-CD8 lineage commitment, developing single positive thymocytes will spend another 4-5 days in the thymic medulla, before they will leave the thymus as mature T cells (Petrie and Zúñiga-Pflücker, 2007). During this time, two distinct mechanisms ensure that the mature T cell repertoire remains tolerant against self-antigens: negative selection and induction of $FoxP3^+$ Tregs. Both of these mechanisms are absolutely essential to prevent fatal autoimmune pathologies (Kyewski and Klein, 2006).

The basis for the seminal theory of negative selection was laid by two independent experiments in the late 80s: First, T cells specific for the MHC molecule I-E are absent from I-E expressing mice (Kappler et al., 1987), and second, transgenic T cells specific for the male antigen H-Y are present in female, but not in male mice (Kisielow et al., 1988). Shortly after that, it was shown that the thymic epithelium is able to mediate deletional tolerance (Salaün et al., 1990), i.e. the deletion of developing thymocytes that recognize self-antigen with a high affinity.

However, how can developing thymocytes be screened for their reactivity with, for example, liver-specific antigens? This remained a puzzle for several years, until it was discovered that medullary thymic epithelial cells (mTECs) have the ability to express antigens that are usually tissue restricted (Derbinski et al., 2001). This phenomenon has been termed "promiscuous gene expression", and subsequent work showed that the transcription factor Aire (autoimmune regulator) is the critical mediator of this process (Anderson et al., 2002). By now it has been clearly established that Aire-dependent expression of a large number of tissue-restricted antigens is essential for immunological tolerance (Anderson et al., 2005), but the mechanism underlying the function of Aire remains under scrutiny (Kyewski and Klein, 2006; Mathis and Benoist, 2009).

In addition to mediating negative selection, the thymus is responsible for a second central tolerance mechanism, namely the generation of $FoxP3^+$ nTregs. Several lines of evidence suggest that at least a fraction of nTregs is selected on the basis of self-antigen-recognition (Jordan et al., 2001), and that co-stimulation (Tai et al., 2005) and cytokine signaling via IL-2 is relevant for this process (Lio and Hsieh, 2008). Both thymic DCs as well as mTECs seem to be equally well equipped to induce nTregs (Wirnsberger et al., 2009), but it remains unknown which mechanism

precisely determines whether a self-reactive thymocyte will undergo clonal deletion or nTreg induction. However, it seems that the number of nTregs is limited to a small thymic niche (Bautista et al., 2009).

γδ T CELL DEVELOPMENT

While for αβ T cells their complex development is understood in remarkable detail, γδ T cell development proved to be more difficult to study, mostly due to the absence of specific markers beside the γδ TCR itself. An early study in the 1990s suggested already that selection of the δ chain occurs roughly at the same developmental stage as β selection, i.e. the DN3 stage (Passoni et al., 1997). However, it was only in 2006 that a novel TCRδ-GFP reporter mouse allowed the definitive proof that the γδ and αβ T cell lineage diverge at the DN3 stage of thymic development (Prinz et al., 2006). Contrary to αβ T cell development there is no pre-γ chain, but rather TCRγ and TCRδ rearrangement occurs simultaneously. It was also shown that the progression of γδ thymocytes beyond the DN3 stage to γδ-TCR^{high} thymocytes is controlled by γδ TCR surface expression, followed by a short proliferative burst (Prinz et al., 2006).

However, while the time-point at which the αβ and γδ T cell lineage diverge has been well defined, the underlying molecular mechanism is still very enigmatic. Up to date, only one transcription factor that promotes γδ T cell development has been defined, namely SOX13 (Melichar et al., 2007), which is a member of the high-mobility group (HMG) family. Overexpression of Sox13 impaired αβ T cell development, while deficiency in Sox13 led to a strongly reduced γδ T cell population. Interestingly, a point-mutation in Sox13 was shown to cause the complete absence of a very specific γδ T cell population, which are IL-17 precommitted Vγ4$^+$ γδ T cells (Gray et al., 2013). This finding further emphasized the importance of Sox13 for γδ T cell development, but additional cell type-specific transcription factors involved in γδ T cell function remain unknown. However, it has been postulated that double positive αβ thymocytes influence the development of γδ T cell precursors *in trans* via LTβ-signals, but the consequences of this interaction have not been understood in detail yet (Silva-Santos et al., 2005).

There are several additional features of γδ T cell development that distinguish them in a remarkable way from conventional αβ T cells: First, in the ontogeny of all vertebrates, γδ T cells mature in the thymus prior to the emergence of the first αβ T cells. This suggests that one important contribution of γδ T cells to immunity is neonatal protection. Indeed it has been reported that in newborn humans γδ T cell are particularly numerous (Gibbons et al., 2009), and that neonatal infection with

Cytomegalovirus (CMV) leads to a strong IFN-γ response by an oligoclonal Vγ8$^+$ pool of γδ T cells (Vermijlen et al., 2010).

Second, while the majority of αβ T cells are thought to leave the thymus as naïve cells, capable of further differentiation towards various effector programs, for γδ T cells thymic pre-programming to a certain effector lineage has been reported. Based on studies of γδ T cells recognizing the non-classical MHC I molecules T10 and T22 (Shin et al., 2005), it was shown that those γδ T cells that have encountered self-antigen in the thymus will become hard-wired towards an IFN-γ producing phenotype, whereas antigen-naïve γδ T cells will turn into IL-17 producers (Jensen et al., 2008). This report was substantiated by the ensuing discovery of a surface marker to distinguish IFN-γ producing from IL-17 producing γδ T cells, namely CD27 (Ribot et al., 2009). Also, certain TCR specificities segregate with the ability to produce a particular effector cytokine, for example Vγ4$^+$ and Vγ6$^+$ γδ T cells mainly comprise IL-17 producing cells. However, the functional implication of this thymic commitment is not yet fully understood. Also, it remains unknown whether γδ T cells require thymic positive selection or not.

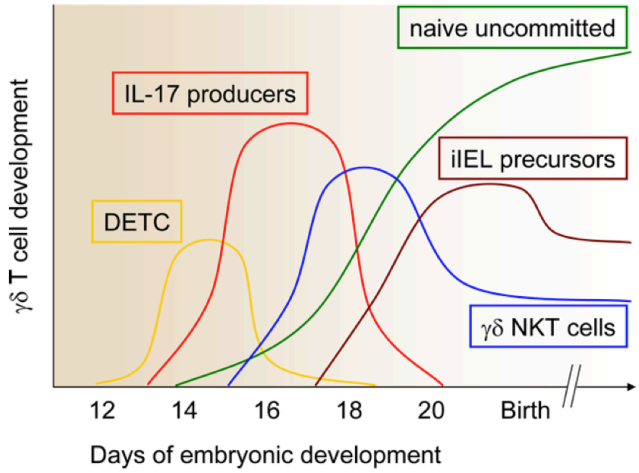

Figure 3: Development of different γδ T cell subsets in ontogeny.

In contrast to αβ T cells, the development of certain γδ T cell populations coincides with hard-wired functional properties and homing receptors. Dendritic epidermal T cells (DETCs) are the very first T cells to develop in the thymus, and also IL-17 producing γδ T cells (mostly of the Vγ4$^+$ and Vγ6$^+$ subclasses) develop only within a certain neonatal window. However, the adult thymus continues to produce γδ T cells (at least in the murine system), whose functional properties remain to be fully characterized. IEL: intraepithelial lymphocyte. NKT: natural killer T cell.
Graph modified from Prinz et al., Eur. J. Immunol 2013.

Third, certain clonal γδ populations develop in embryonic waves and directly populate their respective target organ, with Vγ5$^+$ DETCs being the first mature T cell emerging from the murine thymus (Havran and Allison, 1988).
Further complexity was added by a recent report in that the development of CD27$^-$ IL-17 producing γδ T cells is also restricted to a certain time window during embryogenesis (Haas et al., 2012). This suggests that the thymic microenvironment at a given embryonic stage provides specific cues to developing T cell precursors to steer them towards a particular lineage. However, what processes ensure that despite the vast diversity of theoretical γδ TCR specificities (Carding and Egan, 2002) only few oligoclonal γδ populations develop, remains enigmatic.
In a recent effort by the Immunological Genome Project Consortium, developing murine γδ thymocytes subsets were separated based upon their expression of different TCRγ chains and subjected to whole-genome transcriptome analysis. Surprisingly, this study revealed that the transcriptional program of γδ thymocytes of different specificity diverge from each other as far as for example from CD8$^+$ αβ T cells (Narayan et al., 2012). This emphasizes the notion that thymic pre-commitment is indeed an essential factor in the determination of γδ T cell function, and that the resulting transcriptional networks are vastly different.

DETC DEVELOPMENT

Though dendritic epidermal T cells are only found in the epidermis of mice, these cells due to their identification via the canonical Vγ5Vδ1 TCR provide a unique model system to study general signaling pathways involved during early T cell development as well as the typical properties of intraepithelial lymphocytes (IELs).
As discussed above, Vγ5$^+$Vδ1$^+$ DETCs are the very first T cells to mature in the embryonic thymus (Havran and Allison, 1988) and populate the epidermis, where they remain as a radio-resistant population. The almost monoclonal TCR repertoire of DETCs strongly pointed towards some kind of positive selection mechanism, and indeed in 2008 it was shown that a novel immunoglobulin-like protein termed Skint-1 (Selection and upkeep of intraepithelial T cells 1) is the essential selecting agent (Boyden et al., 2008; Lewis et al., 2006). Skint-1 seems to be specifically expressed by mTECs in the thymus and keratinocytes in the epidermis, but evidence suggests that this molecule does not directly interact with the γδ TCR (Barbee et al., 2011). Notably, the molecular mechanism induced by Skint-1 engagement involves signals via NFκB and NFAT (nuclear factor of activated T cells) and seems to be a conserved pathway also for developing γδ T cells in the adult thymus (Turchinovich and Hayday, 2011).

Unexpectedly, another important role has been attributed to these early γδ T cell precursors by a very recent report showing that $V\gamma5^+$ thymocytes can induce Aire expression in developing mTECs. This interaction involves signaling via the RANK-RANKL (Receptor Activator of NF-κB Ligand) axis (Roberts et al., 2012). However, it remains unknown whether a similar mechanism is in place during human TEC development.

After this overview on the mechanism underlying αβ and γδ T cell development and the generation of tolerance to self-antigens, we switch now to one of the most prominent autoimmune pathologies, which is multiple sclerosis (MS). After that, we will discuss one of the most important signaling pathways in immune cells, NFκB signaling, and its role in the animal model of MS, which is experimental autoimmune encephalomyelitis (EAE). Furthermore, we will outline the known and potential roles of NFκB in the pathogenesis of EAE, but also in the development of γδ T cells and the induction of central tolerance, which is the basis for the data presented within this thesis.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that leads to demyelination of axons and ultimately neurological impairment. The disease is particularly prevalent in Europe and Northern America, and affects approximately twice as many women as men, starting typically at the age between 20 and 40. The most common form of MS is relapsing-remitting MS (RR-MS), which is characterized by sequential clinical episodes that usually resolve themselves over time. RR-MS typically progresses to secondary-progressive MS (SP-MS), during which brain atrophy can be observed (Sospedra and Martin, 2005).

The first description of MS dates back to 1868 by Jean-Martin Charcot, and these days the disease is usually diagnosed by the presence of lesions and plaques in the white matter of the CNS. MS has been considered to be a T cell mediated autoimmune disease, and while the exact etiology as well as the effector mechanism damaging the myelin are still unknown, a combination of environmental risk factors in conjunction with genetic predisposition is held responsible for the development of the disease (Dyment et al., 2004).

Indeed, in 2011 a major genome-wide association study has corroborated the contribution of the immune system to MS pathogenesis: the main risk genes found in this study are involved in cell-mediated immune responses, such as MHC class II molecules, IL-2, IL-12, IL7-receptor and members of the STAT family (Sawcer et al., 2011). In fact the largest risk factors for development of MS are two genes of the HLA-DR15 haplotype (DRB1*1501 and DRB5*0101) (Hillert and Olerup, 1993; Jersild et al., 1973).

Inherently, it is almost impossible to study the cellular events underlying disease development of MS in humans, and hence most of the research in the context of MS relies on animal models. The most prominent among those is experimental autoimmune encephalomyelitis (EAE).

EXPERIMENTAL AUTOIMMUNE ENCEPHALOYMELITIS (EAE)

EAE has been described for the first time more than 50 years ago (Olitsky and Yager, 1949), and ever since then has been used as an animal model to study autoimmune neuroinflammation (McFarland and Martin, 2007). It can be induced by subcutaneous immunization of mice with myelin proteins or peptides in conjunction with complete Freund´s adjuvant (CFA), which induces a myelin-reactive T cell response. The most common antigens used are myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG).

Most basic research has been performed in murine models, due to the good availability of inbred mouse strains and targeted mutations. In C57BL/6 mice, the

disease symptoms start around day 10-12 post immunization with an ascending paralysis, which will first affect the tail, then the hind legs and will proceed towards the front limbs. Correspondingly, inflammation during EAE is mostly seen in the spinal cord and the cerebellum.

Mechanistically, the pathogenesis of EAE has been attributed to auto-reactive T_H1 cells for decades. However, this notion was challenged by the fact that both in mice and men neutralization of the signature cytokine of T_H1 cells, IFN-γ, resulted in exacerbation of EAE and MS symptoms, respectively (Billiau et al., 1988; Panitch et al., 1987a; 1987b) (Chu et al., 2000). Together with the apparent EAE resistance caused by neutralization of the T_H1 polarizing cytokine IL-12 (Leonard et al.), this finding has puzzled immunologists for a while.

This mystery was unraveled by a series of reports in 2003, in which it was shown that the newly discovered cytokine IL-23, which shares the p40 subunit with IL-12, is the critical factor for development of EAE (Becher et al., 2002; Cua et al., 2003).

After the discovery of IL-23 and its link to the polarization of T_H17 cells (Langrish, 2005), a novel scapegoat for autoimmune pathologies was found, namely IL-17. However, while T_H17 cells have been implicated in a variety of autoimmune diseases, including MS (Cua et al., 2003), arthritis (Murphy et al., 2003) and Crohn´s disease (Annunziato et al., 2007), the importance of IL-17 itself in the context of EAE can be questioned (Haak et al., 2008). Indeed, recent reports suggested that pathogenic $CD4^+$ T cells rather show a "multifunctional" state (Hirota et al., 2011), and the current dogma on T helper cell differentiation is changing towards a more "plastic" view (Bluestone et al., 2009). Most importantly, T cell dependent production of granulocyte-macrophage colony stimulating factor (GM-CSF) has been shown to be a critical IL-23 induced effector cytokine during EAE (Codarri et al., 2011; El-Behi et al., 2011).

Taken together, the development of experimental neuro-inflammation seems to be strictly dependent on IL-23, but the exact downstream effector molecules are still a matter of debate, as well as the T cell intrinsic effector mechanisms contributing to the disease. Also, while a lot of research has been devoted to T cell derived effector cytokines, not so much is known about signaling pathways that could modulate disease induction or progression.

THE ROLE OF DCS IN EAE

While T cells without doubt are critical effector cells during EAE, the initial shaping of an adaptive immune response is primarily dependent on the function of DCs (Banchereau and Steinman, 1998). Since their first discovery (Steinman and Cohn, 1973), DCs have been categorized into many different subclasses (reviewed in

(Merad et al., 2013)), the two most important being plasmacytoid DCs (pDCs) and classical DCs (cDCs). cDCs in turn, can again be subdivided into $CD11b^+$ DCs and $CD8^+$ DCs, which in non-lymphoid tissues do not express CD8 but rather CD103 (Ginhoux et al., 2009). $CD8^+$ DCs have been shown to be very efficient in cross-presentation, which is the ability to present phagocytosed antigen in the context of MHC class I molecules (reviewed in (Joffre et al., 2012)).

Several lines of evidence have implicated DCs in the induction and progression of autoimmune diseases, including Type-1 diabetes (Turley et al., 2003), psoriatic skin inflammation (Wohn et al., 2013) and EAE (reviewed in (Ganguly et al., 2013)).

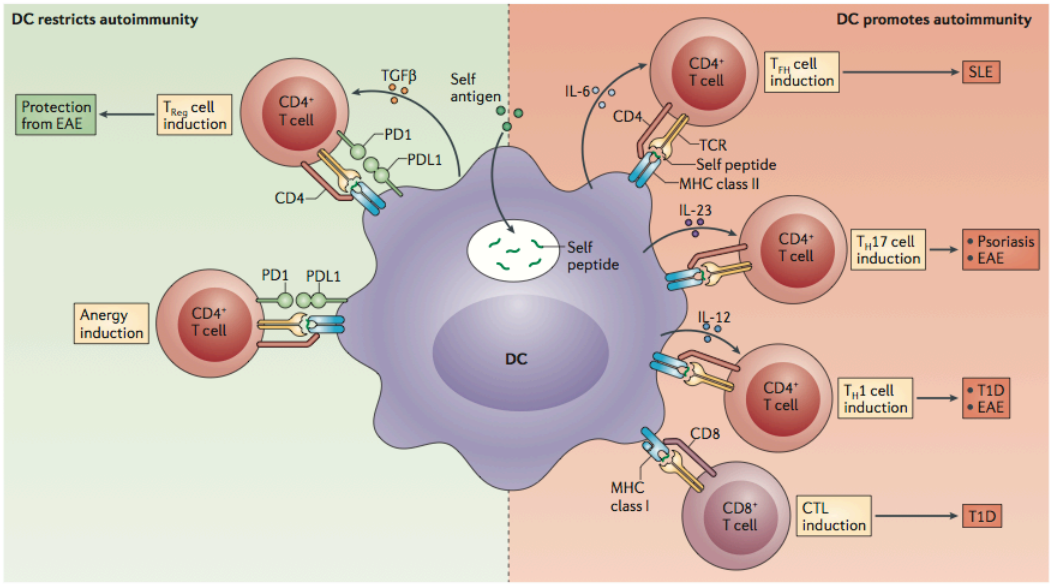

Figure 4: Proposed roles of DCs in autoreactive T cell responses.

There is evidence that during autoimmune reactions, DCs can either have anti-inflammatory (left panel) or pro-inflammatory roles (right panel). In the context of EAE, presentation of myelin antigens in the context of pro-inflammatory stimuli such as IL-23 or IL-12 is the most heralded function of DCs. Particularly secretion of IL-23 might be an essential step in the generation of pathogenic T cells, but it remains unclear what factors determine IL-23 production in DCs. TGFβ: transforming growth factor beta. TCR: T cell receptor. MHC: major histocompatibility complex. CTL: cytotoxic T lymphocytes. IL: Interleukin. PD1: programmed death 1. T1D: Type 1 diabetes. SLE: Systemic lupus erythematosus. EAE: Experimental autoimmune encephalomyelitis.
Graph modified from Ganguly et al, Nat Rev Immunol 2013.

Beside the presentation of self-antigen, the most obvious role of DCs during autoimmunity might be the production of the T cell polarizing cytokines IL-12, IL-23 and also IL-6, making DCs decisive players during initial T cell activation (Oppmann et al., 2000). However, the mechanism governing which of these cytokines will be produced remains incompletely understood. In the context of EAE, it has been

shown that DCs are the only non-redundant cells for the activation of autoreactive T cells (Greter et al., 2005). Also, there has been some evidence that $CD11b^+$ DCs in the CNS are involved in epitope spreading and the production of proinflammatory signals driving T_H17 cells (Bailey et al., 2007), and that CNS-infiltrating myeloid cells are the target of GM-CSF (Codarri et al., 2011). Furthermore, the induction of EAE upon subcutaneous immunization seems to depend on dermal $CD103^+$ DCs (King et al., 2010), which pick up the antigen and migrate to the skin draining lymph node, where they prime the T cell response. However, there are conflicting results as to whether these conclusions can be reproduced in a genetic model that lacks dermal $CD103^+$ DCs (Edelson et al., 2011).

In summary, DCs are without doubt essential cells contributing both to the induction and progression of EAE, but it is only recently that DC-specific signaling pathways involved become unraveled. One example for this is the fact that signaling via the kinase p38α in a DC intrinsic manner is essential for the development of EAE (Huang et al., 2012).

NFκB SIGNALING

Every eukaryotic cell utilizes a complex network of intracellular signaling cascades, which convey signals that are being sensed by cell surface receptors either into the cytoplasm or into the nucleus, thus changing the transcriptional profile of the cell. One of the most prominent signaling pathways is the nuclear factor kappa B (NFκB) pathway, which is utilized by almost all mammalian cell types, but has its most important function in cells of the immune system.

NFκB was discovered in the mid 80s as a positive regulator of kappa light chain expression in B cells (Sen and Baltimore, 1986). By now it is clear that NFκB comprises a family of transcription factors, which play diverse roles in different cells of the innate and adaptive immune system. In mammals, five different family members have been identified: RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) (Li and Verma, 2002). Importantly, these monomeric proteins need to dimerize to form functional NFκB. Various hetero- and homodimers have been identified, explaining the diverse transcriptional changes induced by NFκB signaling.

All monomers share a structurally conserved amino-terminal Rel-homology domain (RHD), in which the dimerization-, the nuclear localization- and the DNA-binding motifs are located. For RelA, RelB and c-Rel the C-terminus contains a transactivation domain. In resting cells, NFκBs are kept inactive by association with cytoplasmic inhibitory proteins, namely inhibitor of NFκB-α (IκBα), IκBβ and IκBε. Furthermore, both NF-κB1 and NF-κB2 are present as larger precursor proteins,

namely p105 and p100, which need to be proteolytically processed for their activation.

Activation of NFκB occurs via many different receptors, but can be divided into two signaling pathways, which have been termed canonical (classical) and non-canonical (alternative) pathway. These pathways integrate different stimuli and will be discussed in further detail below, with the main focus set on the alternative pathway.

CANONICAL NFκB SIGNALING

Among the main activators of the classical NFκB signaling pathway are members of the tumor-necrosis-factor receptor (TNFR) family, IL-1 receptor and members of the toll-like receptor (TLR) family. Downstream of the corresponding receptors are various adaptors, including mainly the TNFR-associated factor (TRAF) proteins. Furthermore, the classical pathway can also be activated via signals from the TCR and BCR, respectively (Su et al., 2002; Sun et al., 2000).

The central protein complex in canonical NFκB signaling is the IKK-complex (also called NEMO), which consists of the two catalytic subunits IKK-α and IKK-β as well as the regulatory subunit IKK-γ. Upon activation, the catalytic subunits phosphorylate IκB-α, which subsequently gets ubiquitylated and degraded, thus releasing the p50-RelA (NFκB1) and p50-c-Rel dimer for translocation into the nucleus. Notably, one of the earliest transcriptional targets of NFκB1 is the gene encoding IκB-α, thus forming a negative feedback loop that prevents irreversible activation of NFκB1 (Sun et al., 1993). The first insights into the role of NFκB1 *in vivo* have been obtained in the mid 90s, when p50 knockout mice were generated (Sha et al., 1995). These animals developed normally, but showed multiple immune defects, in particular impaired B cell responses. Also, control of bacterial infections such as Streptococcus pneumonia was impaired, emphasizing the importance of canonical NFκB activation for initiation of adaptive immunity. Furthermore, NFκB1 knockout animals have been reported to show impaired CD4 T cell responses, leading to reduced clearance of Leishmania major infections (Artis et al., 2003).

NON-CANONICAL NFκB SIGNALING

In 1997, a novel protein kinase was cloned and characterized as a TRAF-2 dependent, positive regulator of NFκB activity (Malinin et al., 1997). This protein was termed NFκB inducing kinase (NIK), also known as Map3k14.

Shortly after that, the so-called alymphoplasia (aly) mutation (Miyawaki et al., 1994) was mapped to the gene locus coding for NIK, suggesting that the phenotype of

NIK$^{aly/aly}$ mice is due to the absence of NIK signaling (Shinkura et al., 1999). The autosomal recessive aly mutation, that arose spontaneously in a breeding facility in 1986, causes very diverse immunological phenotypes: complete absence of lymph nodes and Peyer's Patches, loss of the cortico-medullary border in the thymus, an inability to produce IgA antibodies and a disorganized splenic structure with a loss of B cell follicles.

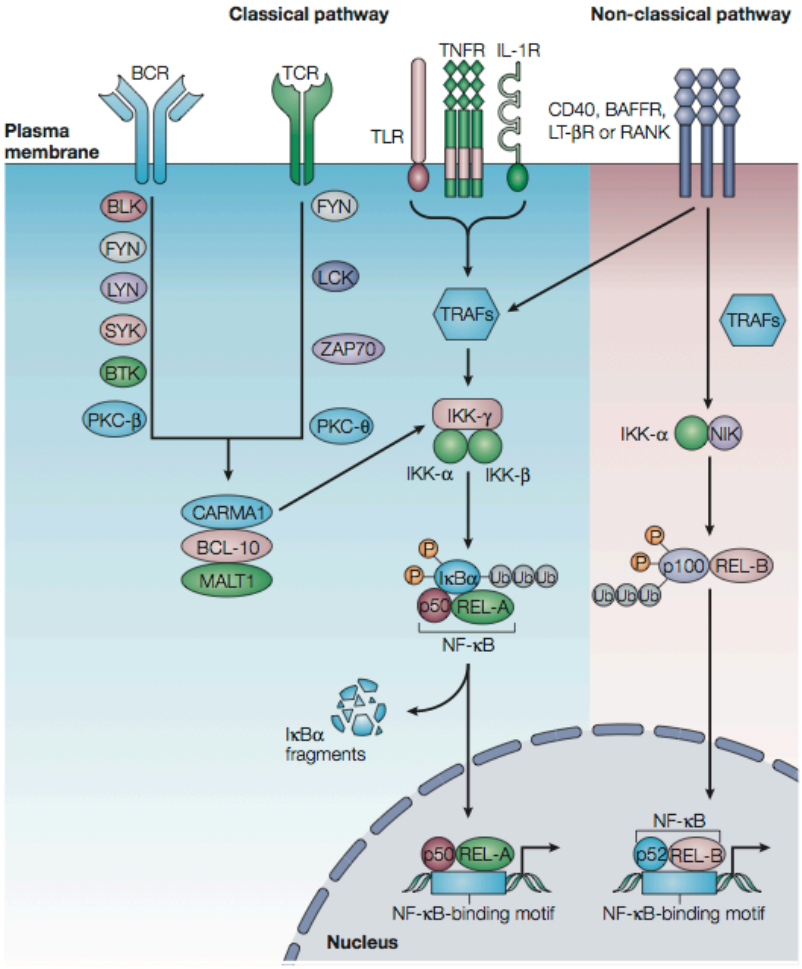

Figure 5: Pathways for induction of NFκB.

The NFκB family of transcription factors is activated by many different receptors, but can be broadly categorized into two signaling pathways: canonical (or classical) NFκB signaling and alternative (or non-canonical) signaling. However, there is evidence for crosstalk between these two pathways.
In the canonical pathway the IKK-α, IKK-β, IKK-γ complex is the central signal integrator. For non-canonical signaling, both IKK-a and the NFκB-inducing kinase (NIK) are essential. Upon activation via upstream TRAFs, NIK protein will get stabilized, phosphorylate IKK-a, which in turn will cause the partial proteolytic processing of p100 to p52. P52 will form a heterodimer with Rel-B and translocate into the nucleus and induce transcription of pro-inflammatory target genes.
BCR: B cell receptor. TCR: T cell receptor. TNFR: Tumor necrosis family receptor. TLR: Toll like receptor. NIK: NFκB inducing kinase.
Graph modified from Siebenlist et al, Nat Rev Immunol 2005.

Final proof that these defects are indeed due to abrogated NIK signaling came from the generation of targeted NIK knockout mice, that exactly phenocopied $NIK^{aly/aly}$ mice (Yin et al., 2001). Subsequently, the importance of NIK as a unique player in NFκB activation was emphasized by a report in 2001, showing that NIK in conjunction with IKK-α can lead to NFκB activation in an IKK-β and IKK-γ independent fashion (Senftleben et al., 2001), thus disconnecting this signaling pathway from the classical IKK(NEMO)-complex. As a result, this novel pathway was termed alternative, or non-canonical NFκB signaling.

The current knowledge on this signaling pathway suggests the following mechanism (reviewed in (Sun, 2010)): in the steady state, NIK is constantly ubiquitinylated and degraded via a TRAF-2/TRAF-3 dependent mechanism (He et al., 2006). Upon surface receptor stimulation, these TRAFs get themselves degraded, leading to the release and accumulation of NIK (Liao et al., 2004). NIK in turn will phosphorylate IKK-α, which will cause the partial proteolytic processing of p100 to p52 (Ling et al., 1998) (Xiao et al., 2001). p52 will form a heterodimer preferentially with Rel-B, translocate to the nucleus and start NFκB2 dependent gene expression. A negative feedback loop via IKK-α mediated NIK phosphorylation exists (Razani et al., 2010).

Of note, signaling via NIK requires *de novo* protein synthesis to allow for accumulation of functional NIK protein (Liao et al., 2004), a feature that distinguishes this pathway from classical NFκB activation that usually proceeds very rapidly without the need for additional protein synthesis.

THE FUNCTION OF NIK SIGNALING IN THE IMMUNE SYSTEM

It has been known for some time that NIK conveys signals from the LT-β receptor (Matsushima et al., 2001) and from the B cell activating factor (BAFF) receptor (Claudio et al., 2002). Hence, non-canonical NFκB signaling has been mainly implicated in lymphoid organogenesis, which is dependent on LT-β (Fütterer et al., 1998), and in B cell maturation and survival (Claudio et al., 2002).

Within the past decade it became evident that signaling via NIK is triggered by additional stimuli, most prominently CD40 (Garceau et al., 2000), Receptor activator of NFκB (RANK) (Darnay et al., 1999), CD28 (Sánchez-Valdepeñas et al., 2006) and CD70 (Ramakrishnan et al., 2004). In addition, some reports challenged the notion that NIK activates only the non-canonical NFκB pathway (Ramakrishnan et al., 2004) (Zarnegar et al., 2008), leaving the open question under which stimuli non-canonical and classical NFκB activation overlap.

However, irrespective of the complex interplay between canonical and alternative NFκB activation, the variety of signals relayed via NIK makes it a key modulator of immune function. Indeed, $NIK^{aly/aly}$ mice have been used for some time as a model

for immunodeficiency, since these animals are unable to mount proper antibody responses, have impaired immunity towards viruses (Karrer et al., 1997) and show a poor ability to reject skin grafts (Lakkis et al., 2000). Initially, this immunodeficiency has been mainly attributed to the absence of secondary lymphoid tissues (SLTs), but as was shown by our lab, SLTs are not a prerequisite for the induction of cell-mediated immunity (CMI) (Greter et al., 2009).

As a result, it became increasingly evident, that beside the lack of SLTs, abrogation of NIK signaling causes intrinsic defects in multiple immune cell types. Indeed, it has been suggested that NIK is critical for full activation of $CD4^+$ T cells and their ability to secrete IL-2 (Ishimaru et al., 2006; Matsumoto et al., 2002). In the study by Ishimaru et al., $NIK^{aly/aly}$ T cells were separated into naïve and memory populations and it was concluded, that naïve $NIK^{aly/aly}$ T cells are in fact hyperresponsive, but suppressed by the $NIK^{aly/aly}$ memory T cell pool via an IL-2 receptor mediated mechanism.

Subsequent to these studies, work from our lab and others showed that both $NIK^{-/-}$ and $NIK^{aly/aly}$ mice are resistant towards the development of EAE (Jin et al., 2009; Greter et al., 2009). Also, we could observe that $NIK^{-/-}$ T cells (in addition to their known impairment to produce IL-2) have a reduced ability to secrete several pro-inflammatory cytokines, including IFN-γ, IL-17 and GM-CSF (Hofmann et al., 2011). Of note, neither in the study by Jin et al., nor in our own work the same proliferative impairments of NIK-deficient T cells were observed that have been reported by Ishimaru et al. previously.

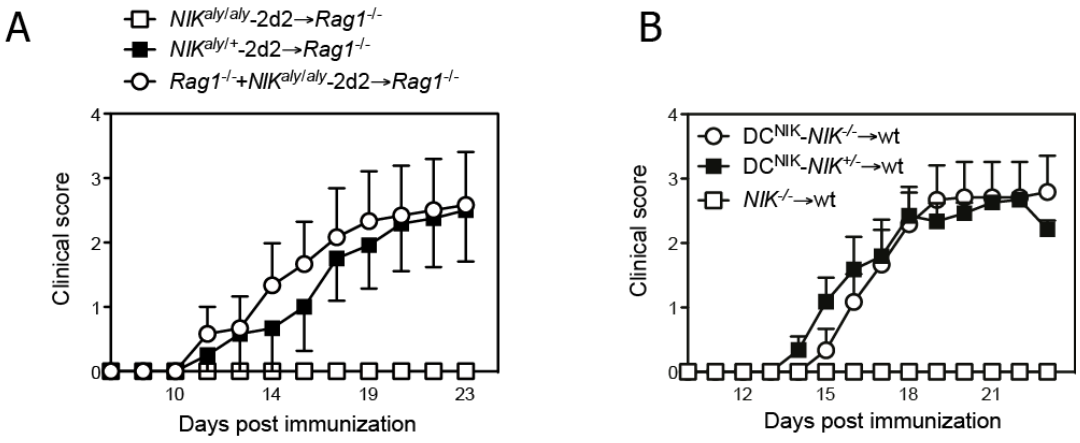

Figure 6: Normal T cell function and susceptibility to EAE is dependent on NIK signaling in DCs, but not in T cells.
(A) Lethally irradiated $Rag1^{-/-}$ mice were reconstituted with bone marrow from $NIK^{aly/aly}$-2D2, $NIK^{aly/aly}$-2D2 or a 4:1 mixture of $Rag1^{-/-}$ and $NIK^{aly/aly}$-2D2 donors. Six weeks after reconstitution animals were immunized with MOG/CFA and monitored for clinical EAE symptoms. **(B)** Lethally irradiated wild-type mice were reconstituted with bone marrow from DC^{NIK}-$NIK^{-/-}$, DC^{NIK}-$NIK^{-/+}$ and $NIK^{-/-}$ donors, and after reconstitution immunized with MOG/CFA. Graphs adapted from Hofmann, Mair et al, J. Exp Med 2011.

However, despite the above mentioned reports that suggested T cell intrinsic defects to be responsible for the EAE resistance of $NIK^{-/-}$ mice (Jin et al., 2009), several experiments performed in our lab indicate that the failure of $NIK^{-/-}$ T cells to initiate cell-mediated immunity and clinical EAE symptoms is rather due to a DC intrinsic defect. First, we have generated a system in which the majority of accessory cells (including DCs) are NIK sufficient, while all T cells are NIK-deficient. To do so, lethally irraditiated $Rag1^{-/-}$ recipients were reconstituted with a 4:1 mixture of $Rag1^{-/-}$ and 2D2 $NIK^{aly/aly}$ bone marrow. 2D2 is the acronym for a TCR transgene encoding a TCR specific for the myelin oligogendrocyte glycoprotein (MOG) (Bettelli et al., 2003). In the resulting chimeras, the majority of the accessory cells will be derived from the $Rag1^{-/-}$ bone marrow, which is NIK sufficient. In contrast, all T cells will be derived from the 2D2 $NIK^{aly/aly}$ bone marrow. In these mixed bone marrow chimeras, normal T cell function is restored (Hofmann et al., 2011). Also, these animals show normal susceptibility to EAE (**Figure 6**a).

Second, we used a transgenic system in which an inducible NIK allele was specifically expressed in DCs, while all other cells remain NIK-deficient. For this, we used a knockin of NIK into the ubiquitously expressed Rosa26 locus (Sasaki et al., 2008) that was preceded by a LoxP-flanked Stop codon. Upon breeding these animals onto a $NIK^{-/-}$ background and further crossing to CD11c-Cre animals, the Stop cassette is excised in $CD11c^{+}$ cells and thus will induce expression of wild-type NIK only in DCs. The resulting DC^{NIK}-$NIK^{-/-}$ mice showed normal susceptibility to EAE, and cytokine production of $NIK^{-/-}$ T cells was restored to wild-type levels (**Figure 6**b) (Hofmann et al., 2011).

Third, we performed experiments in which we adoptively transferred 2D2 T cells into $Rag^{-/-}$ $NIK^{-/-}$ recipients, followed by immunization with MOG/CFA. In this situation, where T cells do not have a genetic defect, but only the APCs including DCs are NIK deficient, no clinical signs of EAE could be observed (Romy Höppli, unpublished data).

All of these experiments strongly argue for an essential DC intrinsic role of NIK during the induction of EAE. In line with this, NFκB2 has been implicated already before in the function of DCs, for example for their ability to cross-present antigens (Lind et al., 2008). Also, LTβ-receptor signaling was shown to regulate the pool size of $CD8^{-}$ splenic DCs (Kabashima et al., 2005). Furthermore, c-Rel was suggested to be involved in TLR-mediated induction of IL-23p19 (Carmody et al., 2007), which is particularly interesting in the context of autoimmune disease.

In summary, it is evident from the available literature that signaling via NIK has critical roles in different immune cell types. Nevertheless, it is not entirely clear yet which cell type depends on the function of NIK for the induction of cell-mediated

immunity, and particularly the role of NIK signaling in different DC populations has not been fully characterized yet.

NIK AND THE ESTABLISHMENT OF CENTRAL TOLERANCE

In addition to these above-mentioned defects in T cells and DCs, a large body of evidence implicates signaling via NIK in the establishment of central tolerance. Already shortly after the initial description of $NIK^{aly/aly}$ mice (Miyawaki et al., 1994) it was reported that despite their generalized immunodeficiency, these animals show chronic inflammatory infiltrates in some exocrine organs such as the salivary gland (Tsubata et al., 1996), suggesting T cell autoreactivity.

As discussed above, central tolerance to a large part is dependent on the promiscuous gene expression of peripheral self-antigens by medullary thymic epithelial cells (mTECs). This process requires expression of Aire (Kyewski and Klein, 2006), and there is still an intense debate about several NIK-dependent signaling pathways that can regulate Aire expression (Mathis and Benoist, 2009).

Initially, it was reported that thymi isolated from LTβ-receptor deficient mice have impaired expression of Aire, causing a mild autoimmune syndrome (Chin et al., 2003; Zhu et al., 2010). However, several subsequent reports questioned this notion (Boehm et al., 2003; Martins et al., 2008; Venanzi et al., 2007). The current view is that the LT pathway does not directly regulate expression of Aire on a per cell basis, but rather is important for the proper spatial organization of mTECs in the thymic medulla (Mathis and Benoist, 2009).

Instead, it seems that another receptor that is signaling via NIK is essential for the development of $Aire^{+}$ mTECs, namely the Receptor Activator of NF-κB (RANK). Signals from RANK ligand (RANKL) derived either from lymphoid tissues inducer cells early in development or from positively selected thymocytes later in ontogeny seem to be essential for the formation of Aire-expressing mTECs (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Rossi et al., 2007). As mentioned in previous chapters, embryonic $V\gamma5^{+}$ γδ T cell precursors might also participate in this process (Roberts et al., 2012).

In summary, several upstream molecules of NIK have been implicated in the formation of a mature thymic mTEC population capable of inducing central tolerance. This would explain why $NIK^{aly/aly}$ and $NIK^{-/-}$ animals in several experimental settings were reported to develop late-onset autoimmune disease (Tsubata et al., 1996) (Kajiura et al., 2004). But numerous open questions remain, among them how in detail NIK signaling influences the maturation of the mTEC pool, which function NIK has in thymic DCs and particularly how the T cell selection process is influenced as a result. While thymic DCs are relevant contributors to negative

selection (Gallegos, 2004), data generated in our lab suggest that they might participate in a "licensing" process that allows developing T cells to reach their full effector potential (Hofmann et al., 2011). This function of thymic DCs seems to depend on NIK and definitely requires further experimentation to reveal the underlying mechanism and relevance for T cell function.

SPECIFIC AIMS

As discussed above, the available data indicate that signaling events via the NFκB inducing kinase (NIK) play distinct roles in various cell types of the immune system, both in the context of central tolerance as well as immune effector function. Due to these manifold phenotypes caused by complete deletion of NIK, the available models make it very tricky (if not impossible) to precisely assess the role of NIK signaling in individual cell types without confounding bystander effects.
Hence, a conditional mouse model is needed to unambiguously clarify the relevance of NIK. This novel tool would clearly broaden our understanding of non-canonical NFκB activation not only in the context of DC and T cell function, but also with regard to the development of lymphoid tissues as well as central tolerance mechanisms.

Within the scope of this thesis I will focus on the following research areas:

- The development and function of lymphoid and tissue-resident γδ T cell subsets in the absence of NIK signaling, using NIK-deficient mice as a model
- The generation and characterization of a novel conditional mouse model ($NIK^{flox/flox}$) that allows tissue-specific deletion of NIK
- Characterizing the effects of DC-specific deletion of NIK on the steady state pool of DCs and the development of cell-mediated immunity, using EAE as a model

In the appendix I will discuss the potential involvement of a recently characterized novel immune cell type, namely innate lymphoid cells (ILCs), during the pathogenesis of EAE.

RESULTS

NIK SIGNALING AND γδ T CELL FUNCTION

DISTURBED DETC COMPARTMENT IN THE ABSENCE OF NIK

As discussed in the introduction, dendritic epidermal T cells (DETCs) expressing the clonotypic Vγ5Vδ1 TCR are the prototype of a highly specialized subset of γδ T cells that only seeds the murine epidermis. Based on previous work from others that has implicated NFκB family members in the development of unconventional iNKT cells (Sivakumar et al., 2003) and the work from our own lab (Hofmann et al., 2011), we speculated that NIK signaling might be necessary for the function of unconventional γδ T cell subsets, of which DETCs are a prime example.

Flow cytometric analysis of the cellular composition of the epidermis in both adult $NIK^{-/-}$ and $NIK^{aly/aly}$ mice revealed striking differences within the DETC compartment. While in control animals, as expected almost 100 % of the $CD45^{+}$ $CD11b^{-}$ cells in the epidermis constitute $CD3^{hi}$ $TCR\gamma\delta^{hi}$ $V\gamma5^{+}$ DETCs, in $NIK^{-/-}$ mice only 30-60% of the cells expressed the Vγ5 TCR (Figure 7b). In addition, an obscure population of $CD3^{int}$ $TCR\gamma\delta^{int}$ was consistently present in $NIK^{-/-}$ mice. To evaluate whether the $V\gamma5^{-}$ cells in $NIK^{-/-}$ animals expressed alternative TCRγ chains, we stained for the Vγ4 and Vγ1.1 chains, which were not detectable (Figure 7c). We also analyzed expression of the Vγ6 chain using the 17D1 clone (Roark et al., 2004), but could not detect any infiltrating $V\gamma6^{+}$ T cells (data not shown). Furthermore, the $V\gamma5^{-}$ cells in $NIK^{-/-}$ animals did not express any TCR β chains (data not shown).

Morphologically, DETCs show a typical dendritic shape very similar to Langerhans cells (Boismenu and Havran, 1994), which has been reported to change upon activation for example by stress ligands (Strid et al., 2008). However, microscopic analysis of epidermal sheets to assess the morphology of Langerhans cells and DETCs in $NIK^{aly/aly}$ animals and littermate controls showed no overt differences both in shape and size (Figure 7d). Also, the density of DETCs in the epidermis appeared similar in $NIK^{aly/aly}$ mice and controls.

Taken together, these findings suggest that in the absence of NIK signaling, an additional $V\gamma5^{-}$ subset of γδ T cells with similar morphologic properties, but unknown TCRγ chain expression populates the epidermis. Additionally, a $TCR\gamma\delta^{int}$ population of unknown origin infiltrates the epidermis of $NIK^{-/-}$ animals.

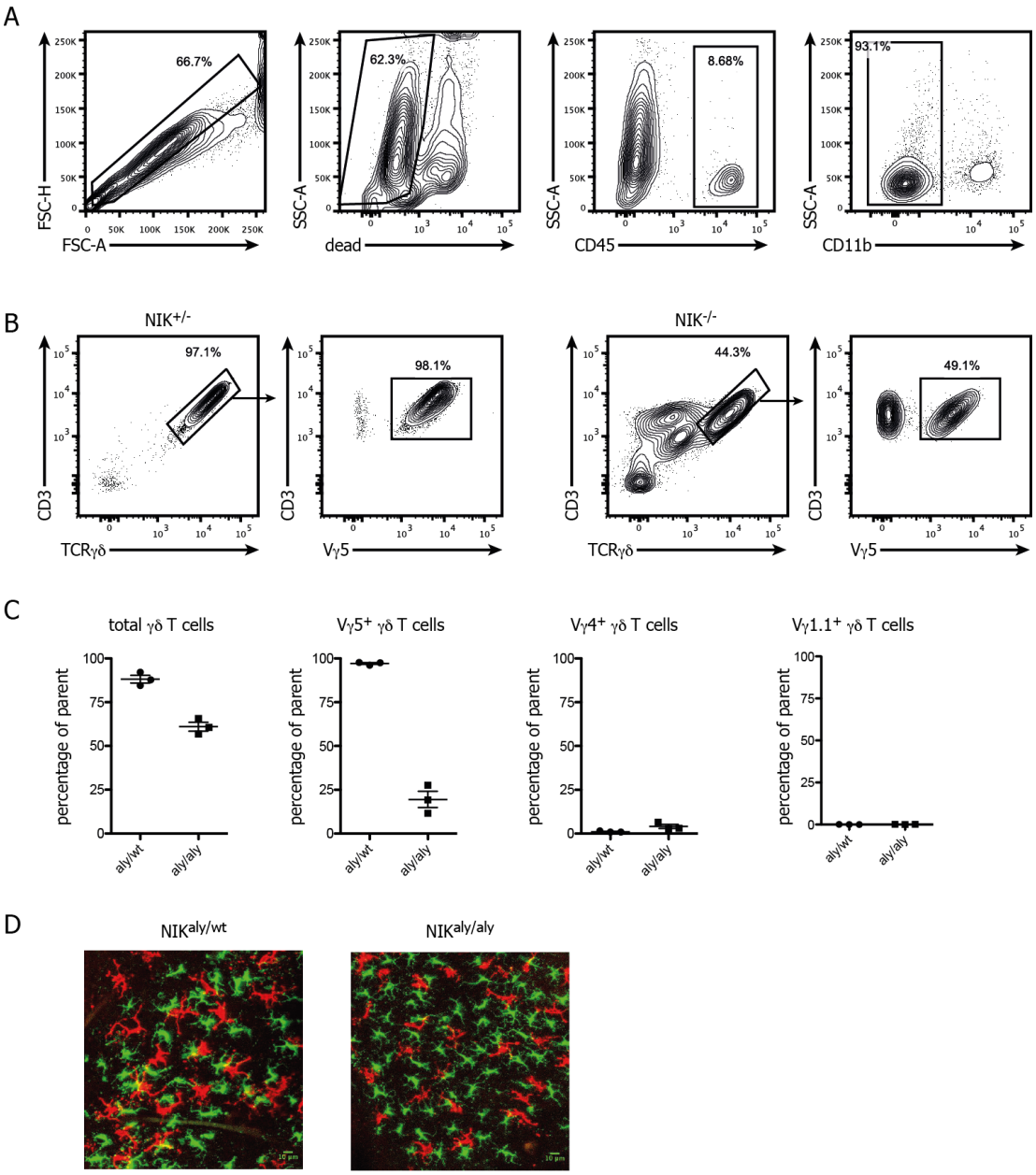

Figure 7: Disturbed steady-state DETC compartment in adult NIK$^{-/-}$ animals.

(A) Gating strategy used for flow cytometric analysis of the epidermis: doublets were excluded by gating on FSC-A vs FSC-H, dead cells were excluded using an amino-reactive live/dead reagent. CD45^{+} CD11b^{-} cells were gated for downstream analysis (similar gating strategies excluding doublets and dead cells were used for all flow cytometric experiments). **(B)** Vγ5 expression within the epidermal γδ T cell compartment in heterozygous controls (left plots) and NIK$^{-/-}$ animals (right plots) **(C)** Quantification of total γδ T cells as well as Vγ5^{+}, Vγ4^{+} and Vγ1.1^{+} γδ T cells in the epidermis of NIK$^{aly/aly}$ mice **(D)** Epidermal sheets were prepared from NIK$^{aly/wt}$ (left plot) and NIK$^{aly/aly}$ ears (right plot) and stained with anti-CD3 (red) to mark DETCs and MHC class II (green) for Langerhans cells.

THYMIC DETC DEVELOPMENT IS BLOCKED IN NIK$^{-/-}$ MICE

Vγ5^{+} DETCs (together with Vγ6^{+} $\gamma\delta$ T cells) are the very first T cells to develop in the embryonic thymus during ontogeny and populate the epidermis already prior to birth (Carding and Egan, 2002; Havran and Allison, 1988). In order to determine whether the observed abnormalities in the adult DETC compartment of NIK$^{-/-}$ mice are due to a developmental defect, we analyzed the epidermis of day 19 embryos.

Surprisingly, we found an almost complete absence of $\gamma\delta$ T cells in the epidermis of NIK$^{-/-}$ animals, while NIK$^{+/-}$ controls had already a sizable fraction of DETCs, which all expressed the canonical Vγ5 chain (Figure 8a). Within the few $\gamma\delta$ T cells found in NIK$^{-/-}$ mice, only around 50% expressed the Vγ5 chain, similar to the steady state situation in adult animals (Figure 8b).

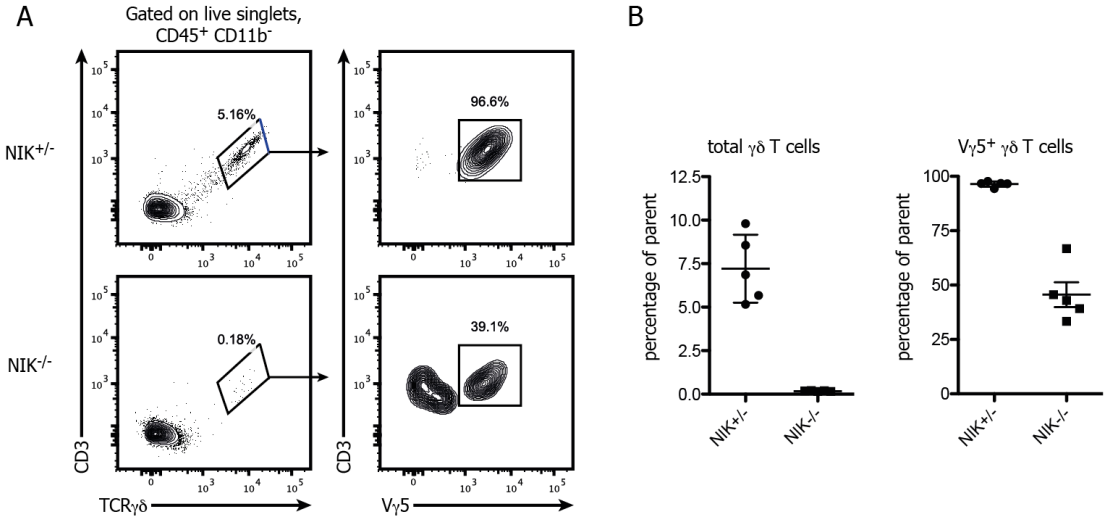

Figure 8: DETCs are absent from the epidermis of embryonic NIK$^{-/-}$ animals.

(A) Flow cytometric analysis of embryonic epidermal sheets on day 19 post conception, pregated on live CD45^{+} CD11b^{-} singlets. Upper row depicts heterozygous controls, lower panel NIK$^{-/-}$ embryos. **(B)** Percentage of total $\gamma\delta$ T cells (left) as well as Vγ5^{+} cells in NIK$^{+/-}$ and NIK$^{-/-}$ embryonic epidermis.

Based on this paucity of $\gamma\delta$ T cells in the embryonic epidermis, we asked whether DETC precursors could be found in the thymus at normal numbers. Indeed, when analyzing the thymi of day 19 embryos, both total $\gamma\delta$ T cells as well as Vγ5^{+} DETC precursors were found in comparable numbers in NIK$^{-/-}$ and NIK$^{+/-}$ animals, albeit the total $\gamma\delta$ compartment appeared to be slightly reduced in NIK$^{-/-}$ thymi (Figure 9a and b). Given the presence of Vγ5^{+} thymic precursors, but the complete absence of mature DETCs in the epidermis, we wanted to assess whether these cells were blocked in their development. Several markers have been associated with the

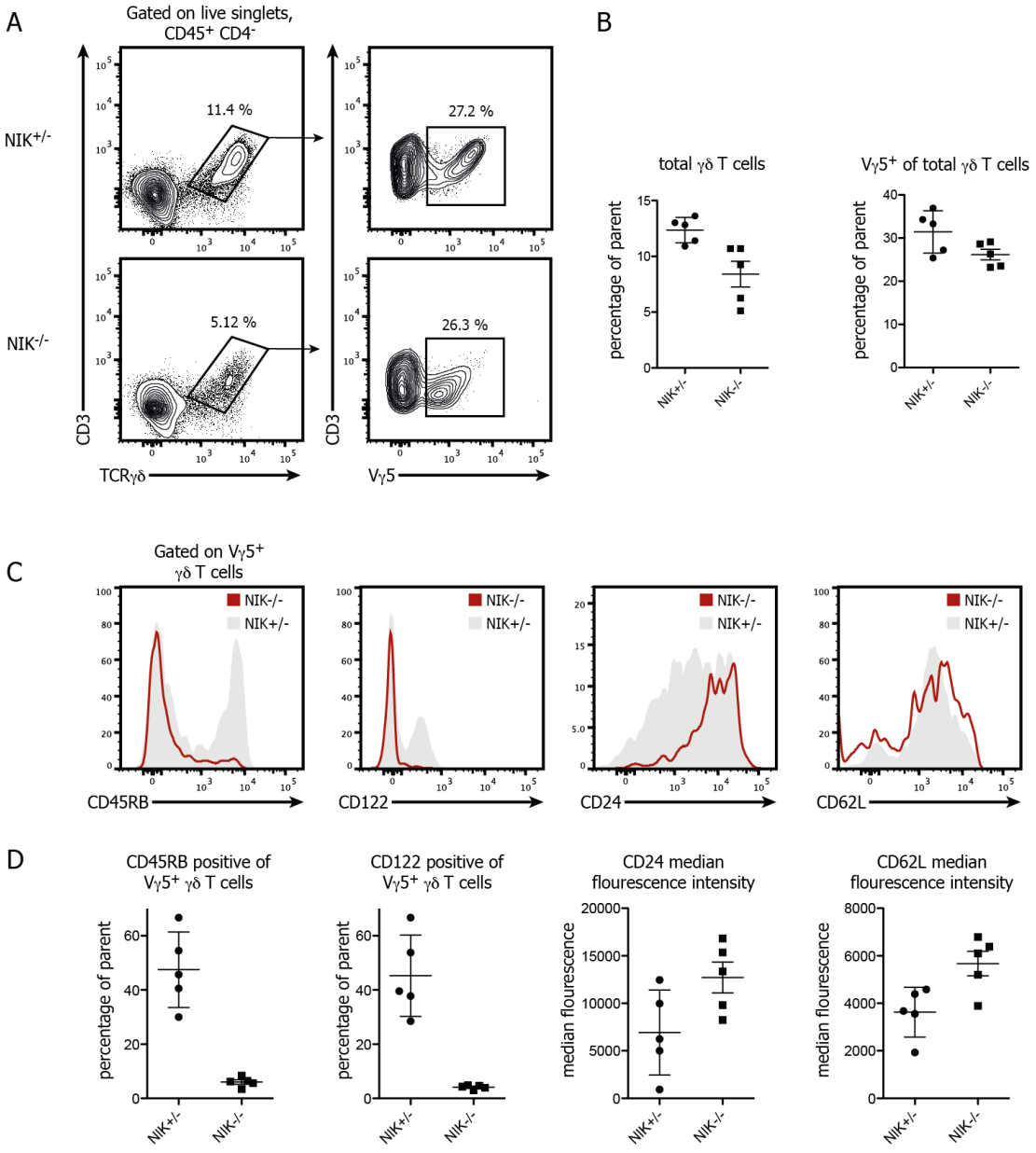

Figure 9: Thymic Vγ5$^+$ DETC precursors seem to be blocked during embryonic development.

(A) Flow cytometric analysis of embryonic thymi isolated on day 19 post conception, pregated on live CD45$^+$ CD4$^-$ singlets. Upper row depicts heterozygous controls, lower row NIK$^{-/-}$ animals. **(B)** Percentage of total γδ T cells (left) as well as Vγ5$^+$ cells in NIK$^{+/-}$ and NIK$^{-/-}$ embryonic thymi. **(C)** Phenotypic analysis of embryonic Vγ5$^+$ thymocytes for expression of CD45RB, CD122, CD24 (HSA) and CD62L. Red histograms shows NIK$^{-/-}$ cells, grey shaded histograms show NIK$^{+/-}$ cells. **(D)** Quantification of CD45RB$^+$ and CD122$^+$ cells within the Vγ5$^+$ compartment in frequency of parent (left two plots) and median fluorescence intensity (MFI) of CD24 and CD62L on Vγ5$^+$ thymocytes (right two plots).

developmental progression of thymic DETC precursors, among them CD24 (also known as heat stable antigen), CD45RB, CD122 and CD62L (Leclercq et al., 1993) (Lewis et al., 2006). Under normal conditions, during their thymic residency from embryonic day 15 till day 19, DETC precursors will upregulate expression of CD45RB and CD122, while CD24 and CD62L levels will be downregulated.

In $NIK^{-/-}$ mice, thymic $V\gamma5^{+}$ cells analyzed on embryonic day 19 failed to upregulate CD45RB and CD122, and also showed reduced downregulation of CD24 and CD62L (Figure 9c and d). The same phenotype was observed when analyzing day 18 embryos (data not shown). Thus, we conclude that in the absence of NIK signaling, embryonic $V\gamma5^{+}$ precursors are developmentally blocked and as a result fail to populate the epidermis. This is similar to the phenotype previously reported in a mutant mouse strain (FVB/N mice from Taconic Laboratories) lacking thymic expression of Skint-1, which is a positively selecting ligand required for developing DETCs (Boyden et al., 2008; Lewis et al., 2006).

THYMIC $NIK^{-/-}$ DETC PRECURSORS ARE REDIRECTED TOWARDS THE IL-17 LINEAGE

Recently, it has been proposed that a molecular mechanism dependent on the transcription factors Egr3, RORγt and Sox13 determines whether developing γδ T cells will progress towards a T-bet dependent IFN-γ producing lineage, or towards an RORγt dependen IL-17 producing lineage (Turchinovich and Hayday, 2011). The default transcriptional program for DETC precursors depends on selection by Skint-1 (Turchinovich and Hayday, 2011), which also causes the upregulation of skin-homing receptors and the subsequent migration to the epidermis (Xiong et al., 2004).

We wanted to test whether NIK deficient DETC precursors not only show a developmental block, but also deviate from their default transcriptional program of T-bet driven IFN-γ production. To do so, we analyzed thymic γδ T cell precursors isolated from d19 embryos for their ability to produce IFN-γ and IL-17.

While for $V\gamma4^{+}$ thymocytes no relevant differences in their cytokine profile could be detected, $V\gamma5^{+}$ DETC precursors isolated from $NIK^{-/-}$ animals showed a marked switch towards an IL-17 producing profile, with up to 50% of cells expressing IL-17 (Figure 10a and b). This was accompanied by a reduced ability for production of IFN-γ. Furthermore, this change in the functional profile of immature DETCs was also detected at embryonic day 18 and in newborn pups (data not shown).

Hence we conclude that in the absence of NIK, developing DETC precursors not only show a developmental block in terms of surface marker expression, but also change their default transcriptional program towards the IL-17 lineage.

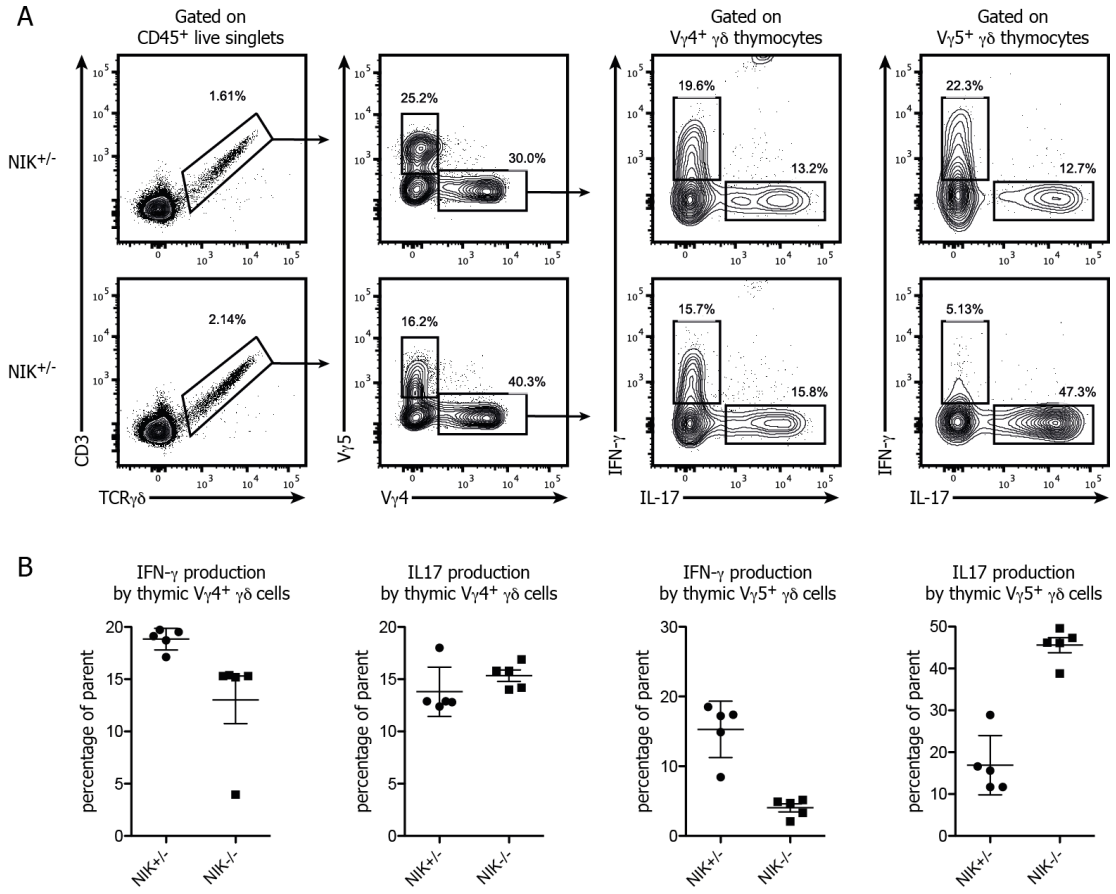

Figure 10: Thymic Vγ5$^+$ DETC precursors in NIK$^{-/-}$ animals are redirected towards the IL-17 producing lineage.

(A) Flow cytometric analysis of Vγ4$^+$ and Vγ5$^+$ thymocytes in day 19 embryos for their ability to produce IFN-γ and IL-17. NIK$^{+/-}$ control animals are depicted in the upper row, NIK$^{-/-}$ animals in the lower row. Left plots are pregated on CD45$^+$ live singlets. **(B)** Quantification of IFN-γ and IL-17 production by Vγ4$^+$ thymocytes (left plots) and Vγ5$^+$ thymocytes (right plots).

SKINT-1 EXPRESSION IS REDUCED IN NIK$^{-/-}$ MTECS

Given that NIK$^{-/-}$ animals have a disturbed thymic architecture with undefined cortico-medullary junction and smaller medulla (Kajiura et al., 2004), one possible explanation for the observed DETC phenotype in NIK$^{-/-}$ thymi could be loss of Skint-1 expression (Boyden et al., 2008; Lewis et al., 2006). Therefore, we sort purified MHC-II$^+$ EpCAM$^+$ mTECs from the thymi of adult NIK$^{aly/aly}$ and NIK$^{aly/wt}$ control animals and analyzed the expression of Skint-1 by qPCR. As has been reported by other groups, fewer mTECs both in frequency and absolute numbers could be recovered from NIK$^{aly/aly}$ thymi than from control animals (Akiyama et al., 2008;

Kajiura et al., 2004). Also, the proportion of mTECs was shifted more towards immature MHC-II low mTECs. Analysis of the Skint-1 expression level showed a roughly 75 % reduction in NIK$^{aly/aly}$ animals compared to controls. Also, expression of Aire was strongly reduced in NIK$^{aly/aly}$ mTECs, which is in line with previous reports showing reduced Aire expression in NFκB2$^{-/-}$ mice (Zhu et al., 2006).

Thus, our results suggest that the observed DETC defects in the absence of NIK signaling are caused by the loss of Skint-1 expression in mTECs. However, we also wanted to test this hypothesis in a genetic model.

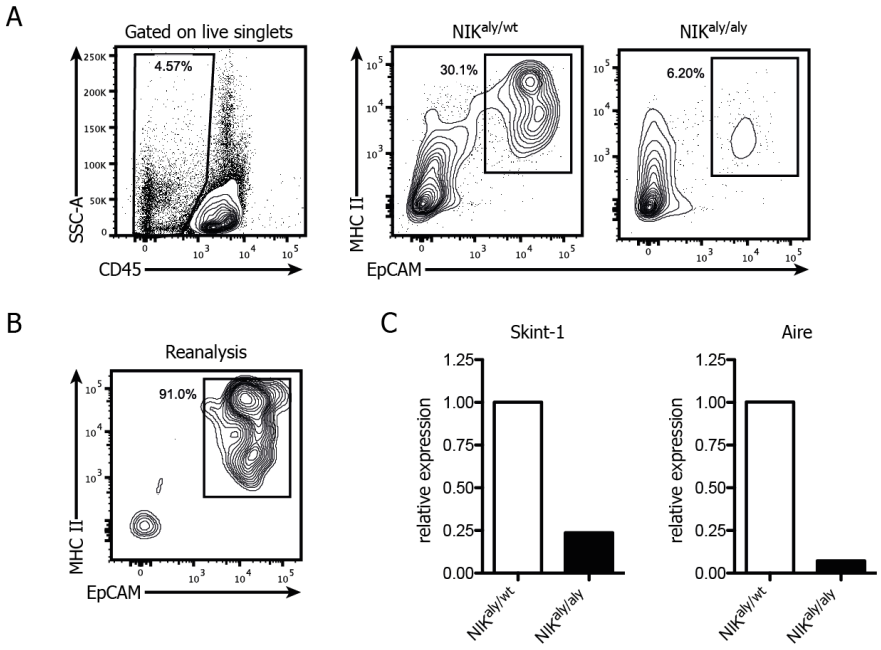

Figure 11: Loss of Skint-1 expression in NIK$^{aly/aly}$ mTECs.

(A) Flow cytometric gating strategy for sorting of EpCAM^{+} MHC-II^{+} mTECs from NIK$^{aly/wt}$ controls (middle plot) and NIK$^{aly/aly}$ animals (right plot). **(B)** Reanalysis of the sorted population. **(C)** qPCR analysis of Skint-1 and Aire expression by mTECs.

DELETION OF NIK IN MTECS DOES NOT INFLUENCE THE DETC COMPARTMENT

The developmental block of embryonic DETC precursors seen in NIK$^{-/-}$ mice could reflect either a cell intrinsic role of NIK signaling in Vγ5^{+} thymocytes, or rather a cell extrinsic mechanism involving medullary thymic epithelial cells (mTECS). Indeed, recent reports suggested a model in which RANK-RANKL interactions between developing γδ thymocytes and immature mTECs are essential for the maturation of Aire^{+} mTECs, which in turn via Skint-1 promote the further development of Vγ5^{+} DETCs (Roberts et al., 2012).

To test this hypothesis, we collaborated with Lucas Onder (Cantonal Hospital St. Gallen), who recently generated a novel BAC-transgenic mouse line expressing Cre recombinase under control of the CCL19 promotor, which is active in LN stromal cells and mTECs (Chai et al., 2013; Misslitz et al., 2004). By crossing this mouse line to the conditional NIK$^{flox/flox}$ strain (see next chapter), NIK should be deleted specifically from the mTEC compartment, but not from γδ T cells. Indeed, preliminary data generated by Lucas Onder suggests that in adult CCL19-Cre NIK$^{flox/flox}$ animals the thymic medulla is almost completely absent.

Analysis of the epidermal immune compartment in adult CCL19-Cre NIK$^{flox/flox}$ animals revealed a normally appearing DETC compartment, both in terms of absolute numbers as well as Vγ5 expression. This would suggest that in contrast to the current belief, γδ T cell intrinsic NIK signaling is required for DETC development.

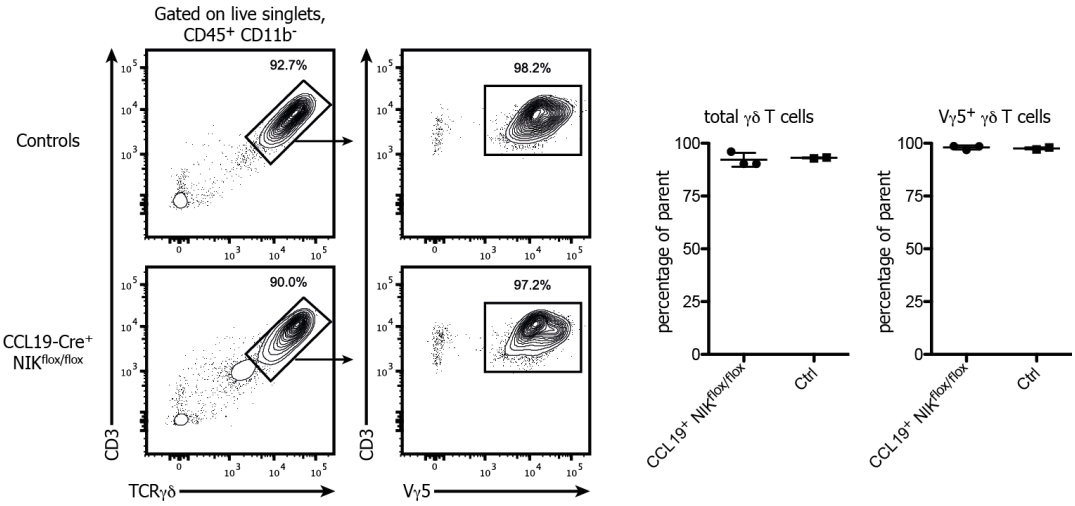

Figure 12: DETC development is not impaired after mTEC specific deletion of NIK using CCL19-Cre NIK$^{flox/flox}$ mice.

Flow cytometric analysis of the epidermal γδ T cell compartment in CCL19-Cre NIK$^{flox/flox}$ mice (lower row) and Cre negative control animals (upper row). Plots are pregated on CD45$^+$ CD11b$^-$ live singlets. Plots on the right show the frequency of total γδ T cells as well as Vγ5$^+$ DETCs.

NIK SIGNALING IS ESSENTIAL FOR IL-17 PRODUCTION BY LYMPHOID γδ T CELLS

Based on the unexpected role of NIK signaling in DETC development we speculated that also other γδ T cell subsets might depend on NIK for their function. Therefore, we aimed at characterizing the composition and functionality of different lymphoid and tissue-resident γδ T cell populations in the absence of NIK signaling.

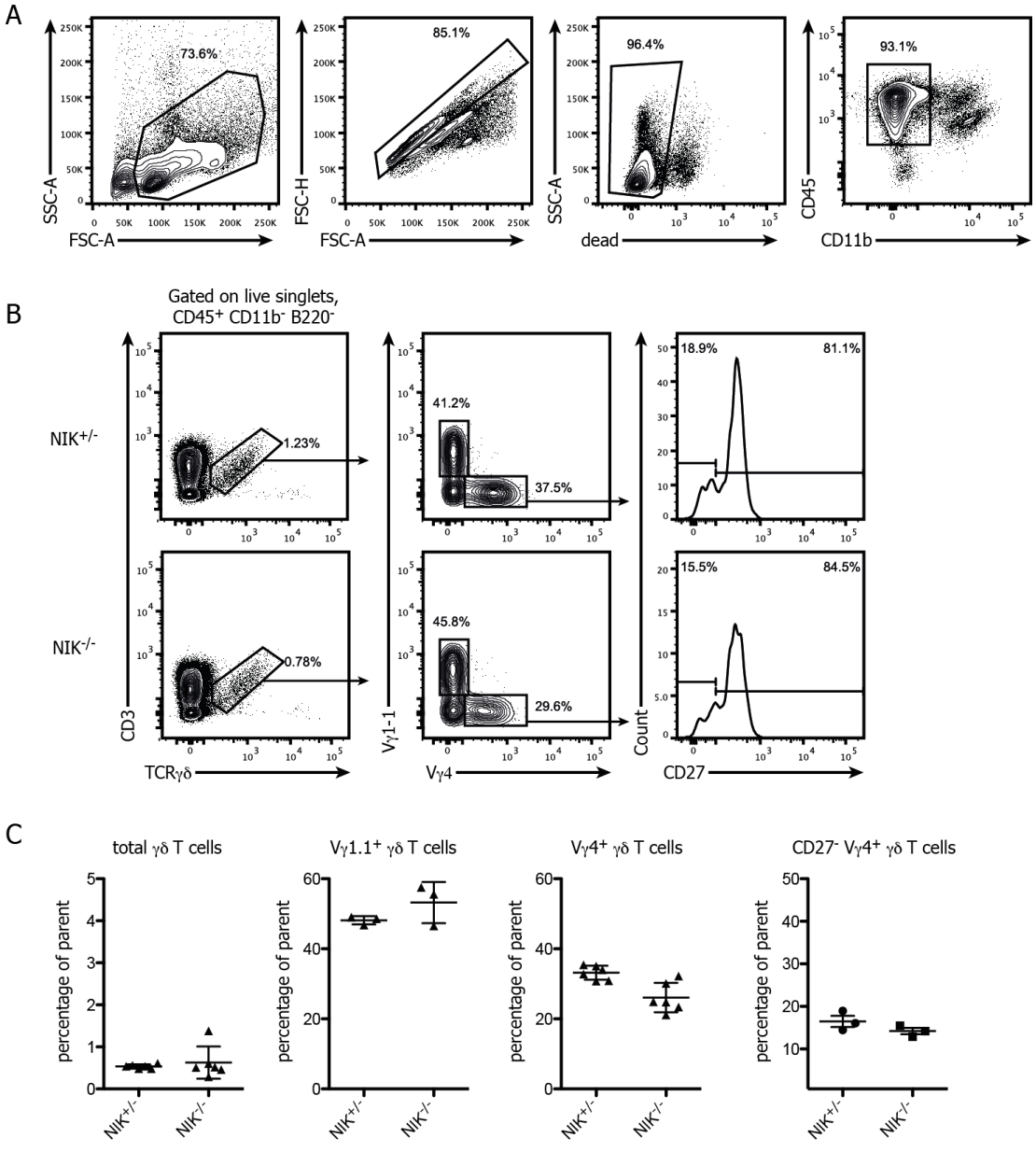

Figure 13: Phenotypic analysis of the lymphoid γδ T cell compartment in NIK$^{-/-}$ mice.

(A) Gating strategy used for analysis of lymphoid γδ T cells. **(B)** Flow cytometric analysis of splenic γδ T cells for the presence of Vγ4^{+} and Vγ1.1^{+} subsets and CD27 expression. **(C)** Quantification of total, Vγ4^{+} and Vγ1.1^{+} γδ T cells, in frequency of parent, as well as CD27^{-} Vγ4^{+} γδ T cells.

In the lymphoid circulation of naïve mice, the majority of γδ T cells can be categorized into two subtypes expressing the Vγ4 and the Vγ1.1 chain, according to the nomenclature of Heilig and Tonegawa (Heilig and Tonegawa, 1986). Furthermore, some γδ T cells are characterized by CD27 expression, which has been correlated with the ability to produce IFN-γ, while $CD27^-$ γδ T cells tend to express IL-17 (Ribot et al., 2009). The majority of IL-17-producers have been shown to express Vγ4 and Vγ6 TCR chains, whereby the latter are restricted to the lung and the uterine tract (Cua and Tato, 2010; Kisielow et al., 2008).

In the spleen of NIK-deficient animals, the proportions of total γδ T cells as well as the $V\gamma4^+$ and the $V\gamma1.1^+$ subpopulations were comparable to heterozygous controls. Also, the distribution of $CD27^+$ and $CD27^-$ cells was similar (Figure 13b and c).

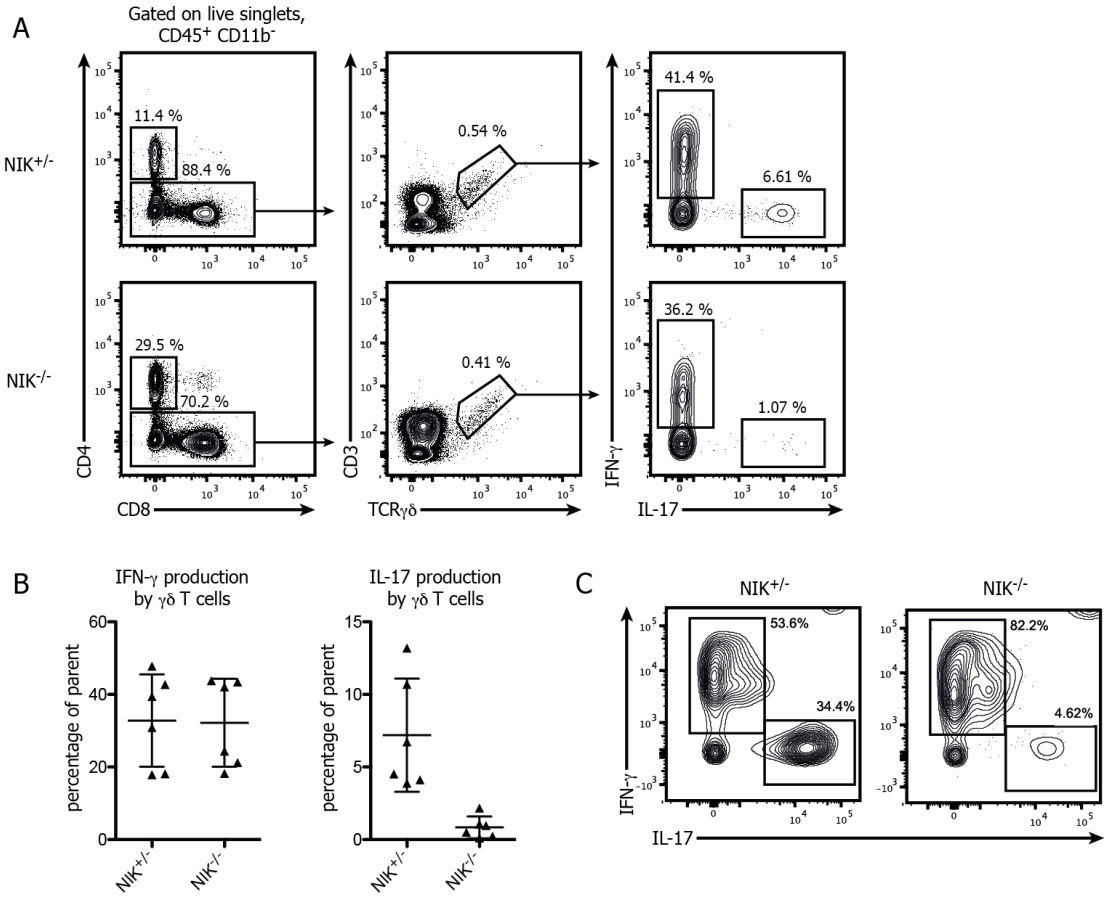

Figure 14: Lymphoid γδ T cells in $NIK^{-/-}$ mice are impaired in IL-17 production.

(A) Flow cytometric analysis of IFN-γ and IL-17 production by *ex vivo* isolated splenic γδ T cells in $NIK^{+/-}$ control (upper row) and $NIK^{-/-}$ animals (lower row). Plots have been pregated on $CD45^+$ $CD11b^-$ live singlets. **(B)** Quantification of cytokine production by splenic γδ T cells. **(C)** Sorted splenic γδ T cells were stimulated for 48 hours with platebound anti-CD3 and IL-2, followed by intracellular cytokine staining. Left plot depicts $NIK^{+/-}$, left plot $NIK^{-/-}$ γδ T cells. Pregating was done on live $CD3^+$ $TCR\gamma\delta^+$ cells.

However, when splenic γδ T cells from NIK$^{-/-}$ mice were assessed for their ability to produce IFN-γ and IL-17 after restimulation with PMA and Ionomycin, we noted that NIK deficient γδ T cells completely lost IL-17 expression, while they maintained the capacity to produce IFN-γ (Figure 14a and b). As has been reported previously, the majority of IL-17 producing cells were confined to the Vγ4^{+} subsets of γδ T cells (data not shown) (Cua and Tato, 2010).

Notably, this inability for IL-17 production remained even after *in vitro* stimulation with αCD3 and IL-2 (Figure 14c), a regimen that has been previously described for activation of γδ T cells (Ribot et al., 2009). This strongly suggests that NIK-signaling is essential for the "thymic imprinting" (Jensen et al., 2008) of γδ T cells towards the IL-17 producing lineage. Very recently, a similar phenotype has been reported to be caused by RelB deficiency in γδ T cells (Powolny-Budnicka et al., 2011). Given that RelB is a downstream target of NIK, this corroborates the notion that non-canonical NFκB signaling is an important driver enabling IL-17 secretion by γδ T cells.

NIK IS ESSENTIAL FOR IL-17 PRODUCTION BY LUNG-RESIDENT Vγ6^{+} γδ T CELLS

Based on the phenotype observed in lymphoid Vγ4^{+} γδ T cells we wanted to assess IL-17 production by another highly specialized γδ T cell subset, namely lung-resident Vγ6^{+} γδ T cells. In ontogeny, these cells develop immediately after Vγ5^{+} DETC precursors, and in contrast to them will migrate preferentially to the lung epithelium and the reproductive tract (Carding and Egan, 2002; Itohara et al., 1990). Vγ6^{+} cells can be identified by the use of the clonotypic antibody 17D1 (Roark et al., 2004).

Steady-state analysis of the lung of adult NIK$^{-/-}$ mice revealed that both Vγ4^{+} and Vγ6^{+} subsets were present, albeit there was a conspicuous reduction in the frequency and staining intensity of Vγ6^{+} γδ T cells (Figure 15a and b). When we assessed their ability for production of IFN-γ and IL-17, Vγ4^{+} cells similar to the situation in the spleen showed strongly reduced IL-17 production, while the level of IFN-γ remained comparable to controls (Figure 15a and c). For Vγ6^{+} cells the situation was more variable, but we also observed a 30-80% reduction in the frequency of IL-17^{+} cells (Figure 15a and c). Notably, these cells were unable to produce any IFN-γ at all.

Therefore, we conclude that in the absence of functional NIK signaling, both Vγ4^{+} and Vγ6^{+} γδ T cells in the periphery and in non-lymphoid tissues are unable to produce pro-inflammatory IL-17. Further work is needed to elucidate the underlying mechanism, in particular whether NIK signaling is needed in a γδ-intrinsic manner, or rather in other (stromal) cell types.

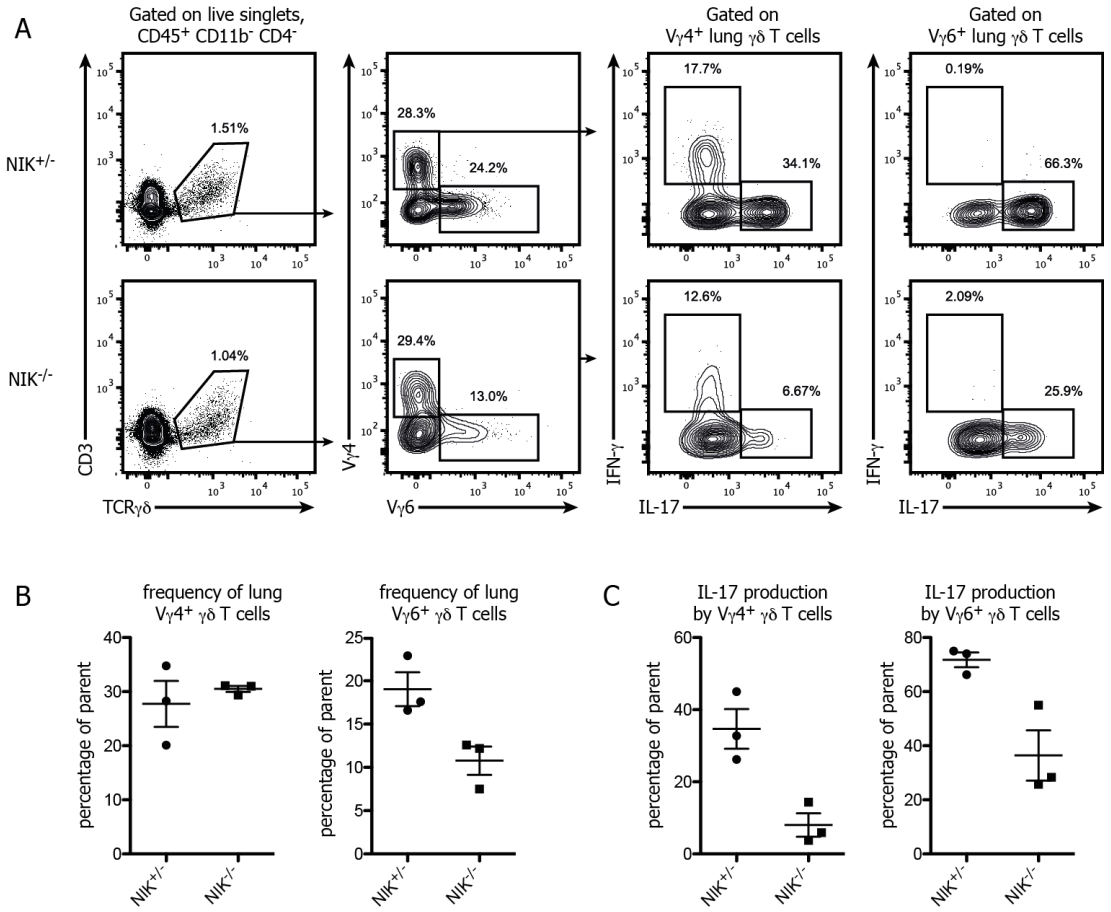

Figure 15: Vγ4$^+$ and Vγ6$^+$ lung γδ T cells in NIK$^{-/-}$ animals are impaired in IL-17 production.

(A) Flow cytometric analysis of IFN-γ and IL-17 production by *ex vivo* isolated lung γδ T cells in NIK$^{+/-}$ control (upper row) and NIK$^{-/-}$ animals (lower row). Plots have been pregated on CD45$^+$ CD11b$^-$ CD4$^-$ live singlets. **(B)** Frequency of Vγ4$^+$ (left plot) and Vγ6$^+$ (right plot) γδ T cells in the lung. **(C)** Frequency of IL-17 producing Vγ4$^+$ (left plot) and Vγ6$^+$ (right plot) γδ T cells in the lung.

NIK SIGNALING AND THE INDUCTION OF CELL-MEDIATED IMMUNITY

GENERATION OF A NOVEL CONDITIONAL MOUSE LINE: $NIK^{FLOX/FLOX}$

As discussed in the introduction, it is by now widely accepted that NIK signaling fulfills diverse roles in different cellular compartments. However, studying the precise function of NIK in individual cell types *in vivo* has proven to be difficult because of the diverse phenotypes found in complete knockout animals. Hence, there is a need for a novel conditional mouse model that would allow cell-type specific deletion of NIK.

Since it was first reported in the 1980s that homologous recombination in murine embryonic stem (ES) cells can be used to genetically target mice (Thomas and Capecchi, 1987), researchers were seeking for a system that would allow tissue-specific gene knockouts *in vivo*. The Cre-LoxP system has been found to be suitable for targeted recombination (Orban et al., 1992; Sauer and Henderson, 1988), and shortly after that the first T cell specific knockout was generated by the Rajewsky lab (Gu et al., 1994).

Loxp sites are 34 base pair long DNA sequences, which are recognized by the bacteriophage P1 derived DNA-recombinase Cre. This recombinase can be expressed under a tissue specific promotor, leading to recombination and thus deletion of the LoxP-flanked target gene only in that particular tissue.

In general, for the generation of mice carrying a LoxP flanked allele, a bacterial targeting vector is generated that contains two homology arms, an antibiotic resistance gene, and at least one essential exon of the gene of interest which is flanked by LoxP sites. This vector is introduced into murine ES cells *in vitro* by electroporation, followed by screening and selection of ES cell clones that have successfully integrated the targeting vector by homologous recombination. These ES cells will then be injected into blastocysts, which after implantation into a pseudopregnant foster mother will give rise to chimeric offspring. These chimeras will consist of a mixture of cells containing either the targeted or the wildtype allele. In case the injected ES cells formed the germ cells of these chimeric animals, the F1 offspring will carry the conditional allele and can be further bred to homozygosity.

In an initial attempt to generate a conditional NIK allele, we designed and cloned a vector targeting exon 3 of the NIK genomic locus (also known as Map3k14 locus), which was verified by restriction digests and sequencing (data not shown). However, after electroporation and selection of JM8N6 ES cells, the screening of more than 600 ES cell clones both by Southern Blot and long range PCR did not yield any homologously recombined clones (data not shown) and hence we abandoned this approach.

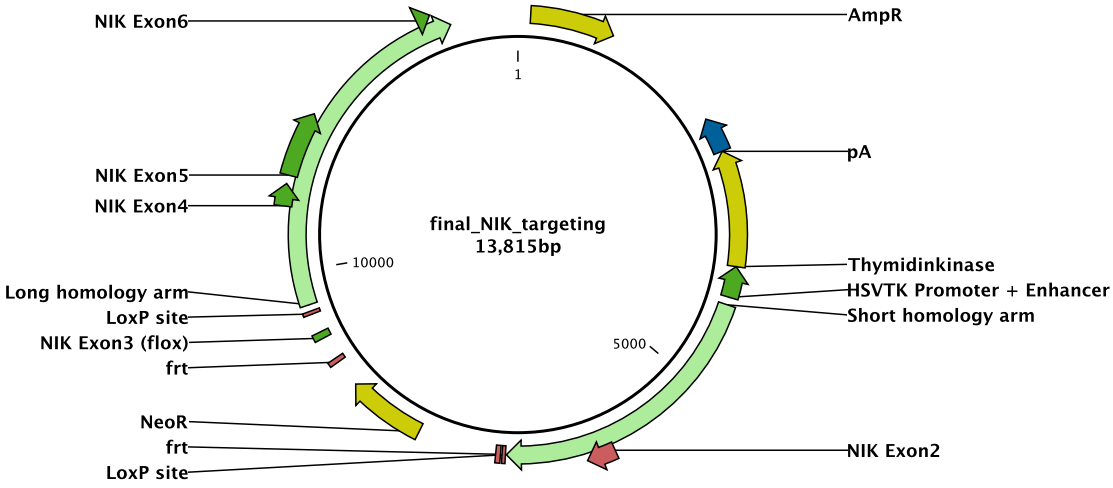

Figure 16: Targeting vector used for the initial generation of a conditional NIK allele in ES cells.

The targeting vector consisted of a short and long arm of homology, the frt flanked Neomycin resistance (NeoR) cassette, the LoxP flanked Exon 3 and the thymidine kinase gene used to reduce the number of ES cell clones integrating the vector at a random site in the genome. The restriction sites used for the Southern Blot screening of the ES cell clones are not depicted.

Therefore, in collaboration with the lab of Ari Waisman in Mainz (Germany) and Taconic-Artemis in Cologne (Germany) an alternative targeting strategy was designed, which employed slightly different homology arms, thereby targeting Exons 4-6 of NIK. Using this approach, targeted ES cells as well as chimeric animals carrying the conditional allele ($NIK^{flox/wt}$) were successfully generated by Taconic-Artemis. These mice were crossed to Flp-deleter animals to remove the Neomycin resistance cassette, followed by breeding to homozygosity and to two different Cre lines: CD4-Cre, which would delete NIK in all $\alpha\beta$ T cells (Wolfer et al., 2001), and CD11c-Cre, which would delete NIK in all dendritic cells (Caton et al., 2007; Stranges et al., 2007). Furthermore, in collaboration with Lucas Onder from the Cantonal Hospital of St. Gallen, $NIK^{flox/flox}$ mice were crossed with a CCL19-Cre line, which is active in lymph node stromal cells as well as medullary thymic epithelial cells (Chai et al., 2013).

CD11c-Cre $NIK^{flox/flox}$ and CD4-Cre $NIK^{flox/flox}$ mice were born at normal Mendelian ratio and appeared fertile and healthy without obvious defects. Of note, in contrast to the complete NIK knockout, both lines showed normal presence of lymph nodes. In order to assess the functionality of the conditional allele and the deletion efficiency after Cre-mediated recombination, we sort-purified $CD4^+$ T cells,

$CD11c^{+}MHC\text{-}II^{HI}$ DCs and $CD19^{+}$ B cells from the spleen of CD11c-Cre $NIK^{flox/flox}$, CD4-Cre $NIK^{flox/flox}$ and littermate control animals (Figure 18a). Reanalysis of the sorted populations confirmed their purity to be routinely above 95% (Figure 18b). Following RNA isolation and cDNA synthesis, we performed quantitative real-time PCR (qPCR) analysis for the expression level of NIK mRNA.

In T cells obtained from CD4-Cre $NIK^{flox/flox}$ animals, the expression of NIK mRNA was more than 90% reduced compared to control animals, while there was no change neither in B cells nor in DCs (Figure 18c). In contrast, CD11c-Cre $NIK^{flox/flox}$ mice showed the same expression level of NIK in T cells and B cells, while in DCs the expression was reduced approximately 70-80%, indicating that deletion took place.

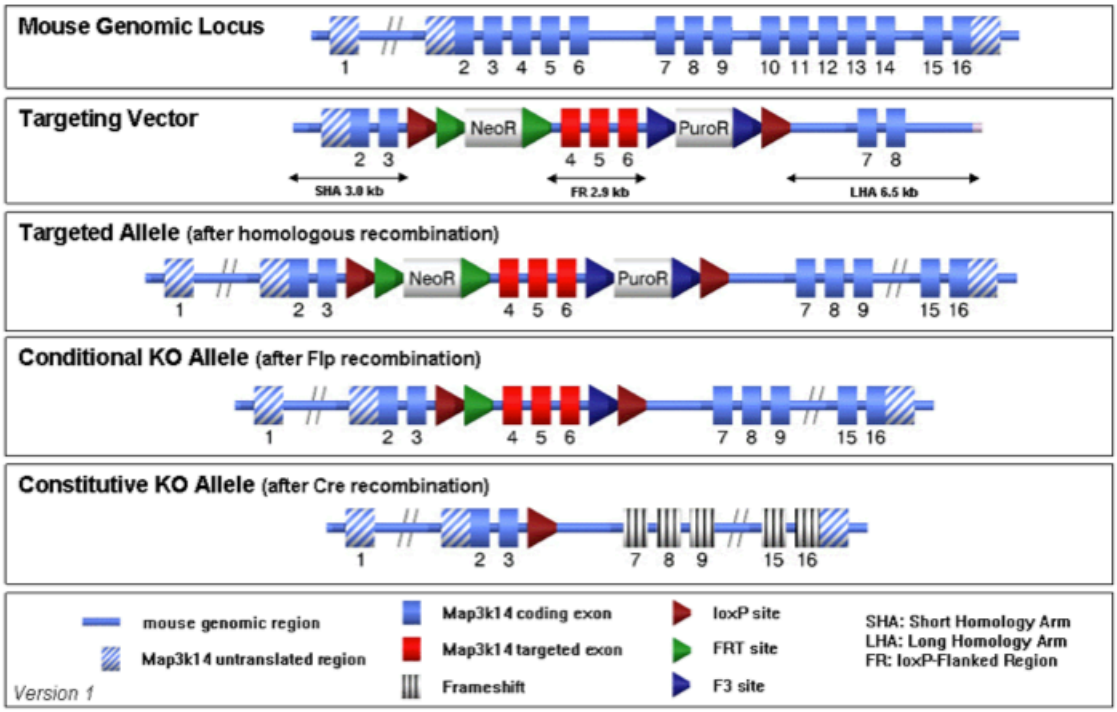

Figure 17: Targeting strategy used by Taconic-Artemis to generate a conditional NIK knockout allele.

Upper row depicts the genomic locus of NIK (Map3k14), with 16 known exons (blue) and the untranslated regions (shown in shaded blue). The translation initation codon for NIK is situated in Exon 2. Second and third row show the targeting vector and the genomic locus after homologous recombination in ES cells.

After Flp mediated recombination the Neomycin and Puromycin resistance genes will be excised, leaving Exons 4-6 directly flanked by LoxP sites. Cre mediated recombination will produce a frameshift in all subsequent Exons, leading to a premature Stop Codon in Exon 7, which will target the resulting transcript for nonsense mediated RNA decay.

(Graphic taken and modified from Taconic-Artemis)

Based on this data we assumed that the conditional NIK allele was functional, and that deletion in T cells and DCs occurred at a good efficiency. However, given the incomplete deletion in DCs, we crossed NIK$^{flox/flox}$ mice to another CD11c-Cre-line obtained from the lab of Boris Reizis (Columbia University, New York, USA), which has been reported to be more efficient (Caton et al., 2007). Unless stated otherwise, subsequent experiments have been performed with the latter line.

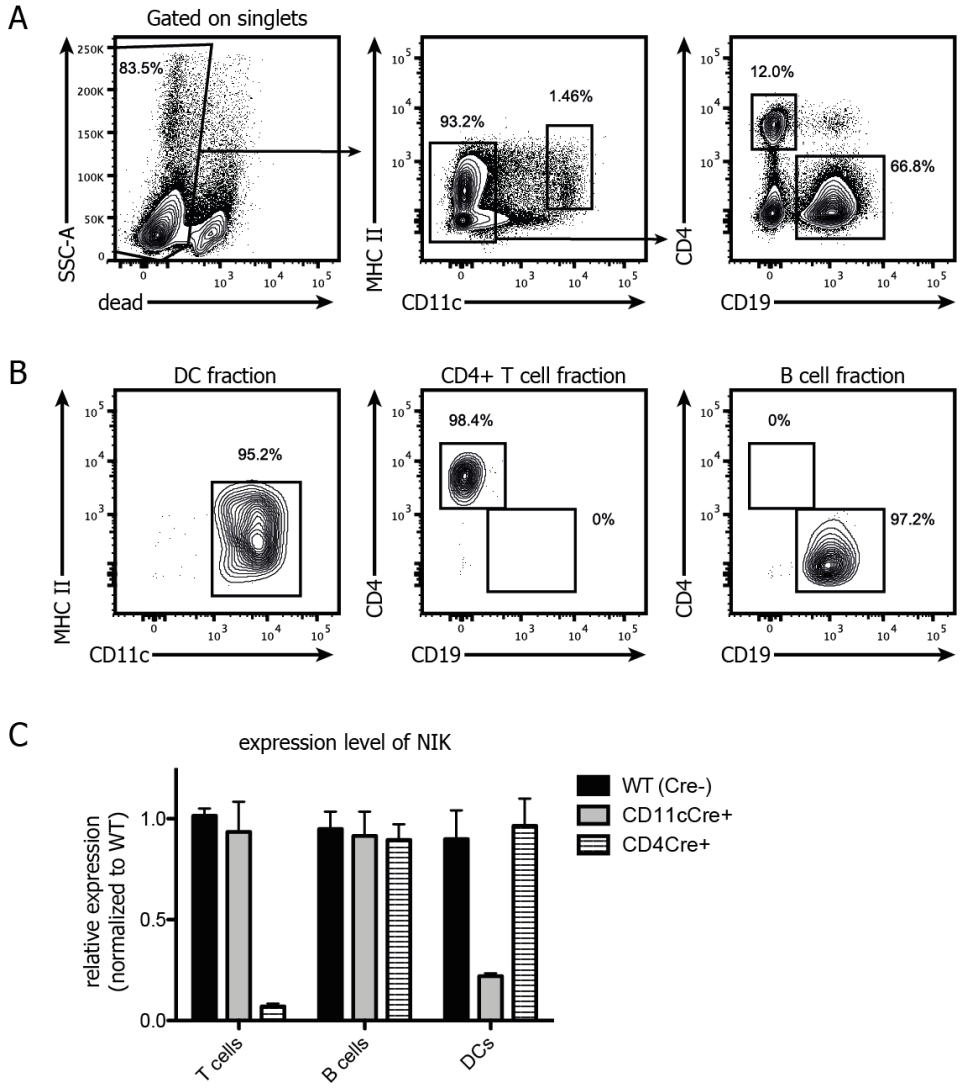

Figure 18: Deletion of NIK in CD4-Cre NIK$^{flox/flox}$ and CD11c-Cre NIK$^{flox/flox}$ mice occurs at a good efficiency.

(A) Gating strategy used to sort CD11c^{+} MHC II^{+} DCs, CD4^{+} T cells and CD19^{+} B cells from the spleen. **(B)** Reanalysis of sorted populations (no upstream gating beside a FSC threshold was applied). **(C)** qPCR analysis of sorted populations for the expression level of NIK in wildtype controls (black bars), CD11c-Cre NIK$^{flox/flox}$ (grey bars) and CD4-Cre NIK$^{flox/flox}$ cells (grey shared bars). Expression level of the WT has been assigned the value of 1.

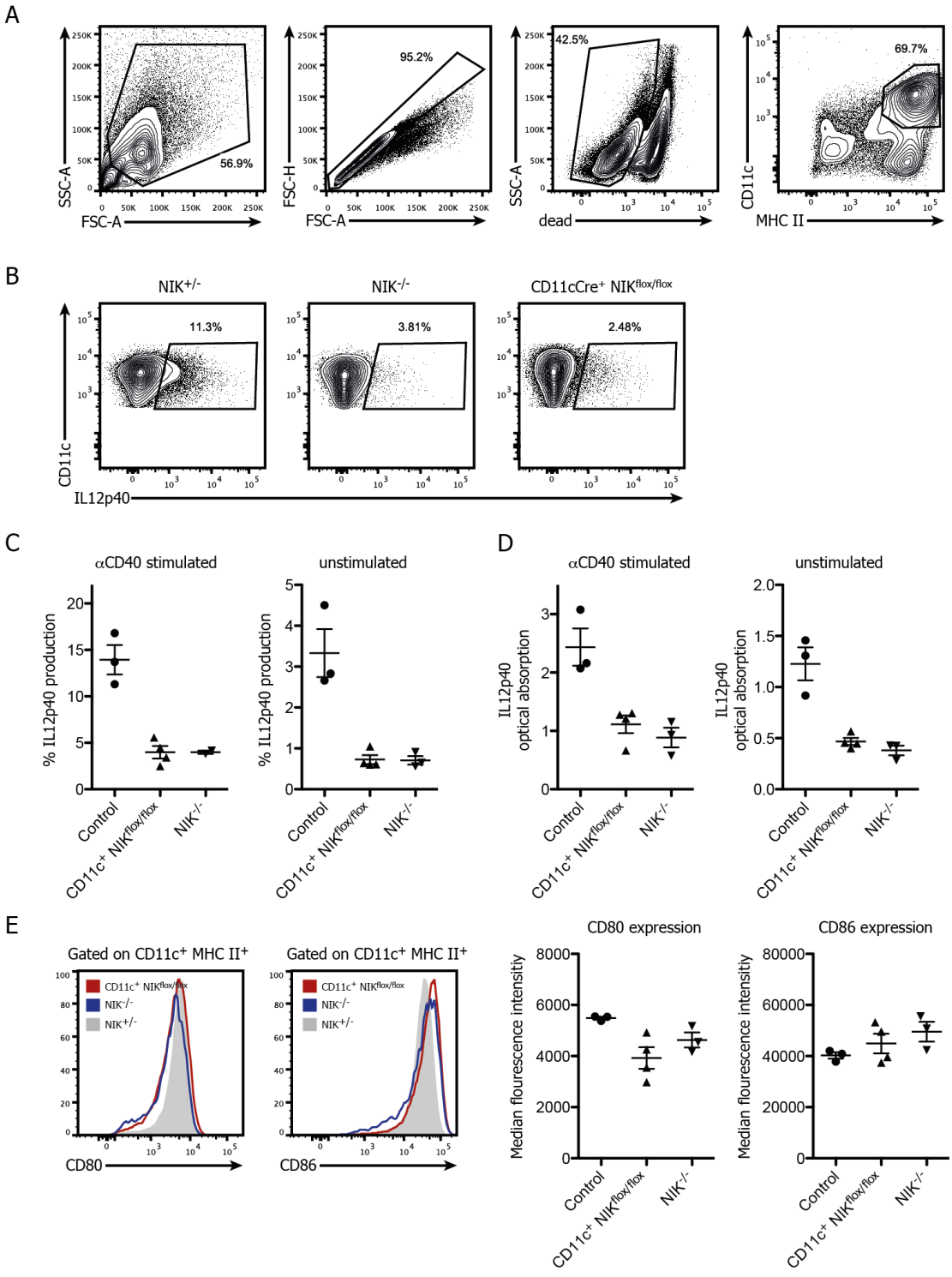

Figure 19: DCs in CD11c-Cre^{+} NIK$^{flox/flox}$ mice show impaired IL-12/23p40 production.

Splenic DCs were isolated by magnetic enrichment using CD11c-Microbeads (clone N418) and stimulated with αCD40 antibodies for 16 hours. For the last 4 hours of stimulation, Brefeldin A was added. **(A)** Gating strategy used for intracellular IL-12/23p40 staining in CD11c^{+} MHC-II^{+} DCs. **(B)** IL-12/23p40 expression by NIK$^{+/-}$ control (left), NIK$^{-/-}$ (middle) and CD11c-Cre^{+} NIK$^{flox/flox}$ (right) DCs. **(C)** Quantification of IL-12/23p40 positive cells after αCD40 stimulation (left plot) or of unstimulated (right plot) cells. **(D)** Quantification of IL-12/23p40 in the supernatant by ELISA. **(E)** Median fluorescence intensity of CD80 and CD86 expression by stimulated CD11c^{+} MHC-II^{+} DCs.

FUNCTIONAL CHARACTERIZATION OF THE DC-SPECIFIC DELETION OF NIK

Several reports from our lab (Hofmann et al., 2011) and others (Lind et al., 2008) suggested that NIK signaling seems to play a particularly important role in the function of DCs. CD40, which is a main activating receptor for DCs, has been shown to signal via NIK (Garceau et al., 2000), and NFκB2 activation has been implicated in the ability of DCs for cross-presentation (Lind et al., 2008).

To clarify whether the conditional allele is fully functional and whether DCs in CD11c-Cre $NIK^{flox/flox}$ animals resemble some of the phenotypes described in complete knockouts, we MACS purified splenic $CD11c^{+}MHC\text{-}II^{+}$ DCs from CD11c-Cre $NIK^{flox/flox}$, $NIK^{-/-}$ and control animals and stimulated them *in vitro* overnight with agonistic αCD40 antibodies. After that, we assessed the expression of CD80, CD86 and MHC class II as well as the ability for IL12p40 secretion by intracellular cytokine staining.

While there was no detectable difference in the expression level of costimulatory molecules such as CD80 and CD86, DCs from CD11c-Cre $NIK^{flox/flox}$ secreted significantly less IL12p40 as revealed by intracellular cytokine staining (Figure 19b and c), similar to the phenotype previously reported for $NIK^{-/-}$ animals (Hofmann et al., 2011). This difference was already detectable without stimulation via CD40. To verify the ICS data, we collected supernatants from the *in vitro* cultured DCs and performed ELISA for IL12p40. Both in anti-CD40 stimulated as well as in unstimulated samples the amount of p40 in the supernatant was significantly decreased in CD11c-Cre $NIK^{flox/flox}$ and $NIK^{-/-}$ DCs compared to controls (Figure 19d).

Thus, we conclude that DC-specific deletion of NIK impairs the ability of splenic DCs to secrete pro-inflammatory IL-12. Notably, this defect is not only noticeable after anti-CD40 stimulation, but also in unstimulated DCs. Based on these phenotype we conclude that the conditional allele is working as expected.

As a next step, we wanted to analyze the steady state T cell compartment of CD11c-Cre $NIK^{flox/flox}$ for any irregularities. Flow cytometric analysis of splenocytes showed that a normal frequency of CD4 to $CD8^{+}$ αβ T cells, and also the ratio of $CD62L^{+}$ $CD44^{-}$ naïve to $CD62L^{-}$ $CD44^{+}$ T cells was comparable between CD11c-Cre $NIK^{flox/flox}$, $NIK^{-/-}$ and control animals (Figure 20a and c). $FoxP3^{+}$ Tregs were found in similar frequencies in CD11c-Cre $NIK^{flox/flox}$ and control animals, but in lower numbers in $NIK^{-/-}$ mice, as reported previously (Hofmann et al., 2011). Also, the γδ T cell compartment appeared normal in terms of absolute numbers and expression of CD27 (Figure 20a and c). Hence we conclude that DC-specific deletion of NIK does not lead to any obvious disturbance of the T cell compartment.

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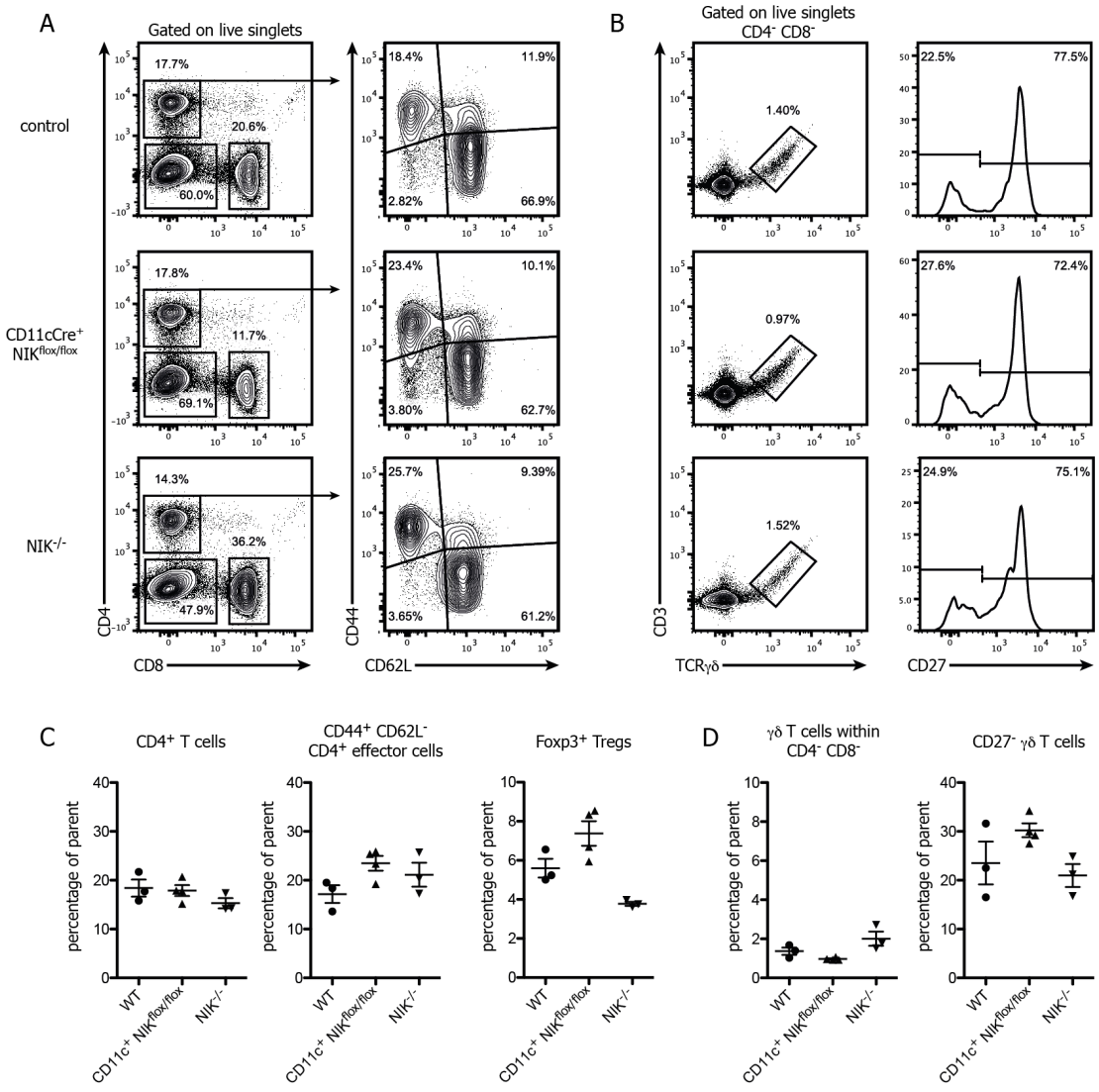

Figure 20: The steady state T cell compartment of CD11c-Cre^{+} NIK$^{flox/flox}$ animals appears phenotypically normal.

(A) Flow cytometric analysis of the frequency of CD4^{+} T cells and CD44^{+} CD62L^{-} effector T cells in the spleen of control (upper row), CD11c-Cre^{+} NIK$^{flox/flox}$, and NIK$^{-/-}$ animals. **(B)** Flow cytometric analysis of the γδ T cell pool. **(C)** Summary of the frequency of of CD4^{+} T cells and CD44^{+} CD62L^{-} effector T cells as well as FoxP3^{+} Tregs. **(D)** Summary of the frequency of total and CD27^{-} γδ T cells.

After we had clarified that the conditional allele is working and does not cause any obvious disturbances in the T cell compartment, we sought to clarify whether the DC compartment of CD11c-Cre NIK$^{flox/flox}$ animals appears normal. For this, we focused particularly on the DC populations in the lymph node because it is not possible to study these in straight NIK$^{-/-}$ mice due to the complete absence of lymph nodes. Up to now, several distinct populations of DCs have been identified in skin-draining LNs:

the MHC-II^{low} DC compartment can be separated into CD8^{+} and CD11b^{+} conventional DCs (cDCs), and the MHC-II^{high} compartment contains Langerin^{-} and Langerin^{+} DCs, the latter representing DCs that migrate from the skin to the draining LN (Henri et al., 2001; Randolph et al., 2005; Salomon et al., 1998; Stoitzner et al., 2003). When we assessed the steady state LN compartment of CD11c-Cre NIK$^{flox/flox}$ animals, we found similar proportions of MHC-II^{high} and MHC-II^{low} DCs (Figure 21a and b). Also the frequency of CD11b^{+} and CD8^{+} cDCs was comparable to control animals (data not shown).

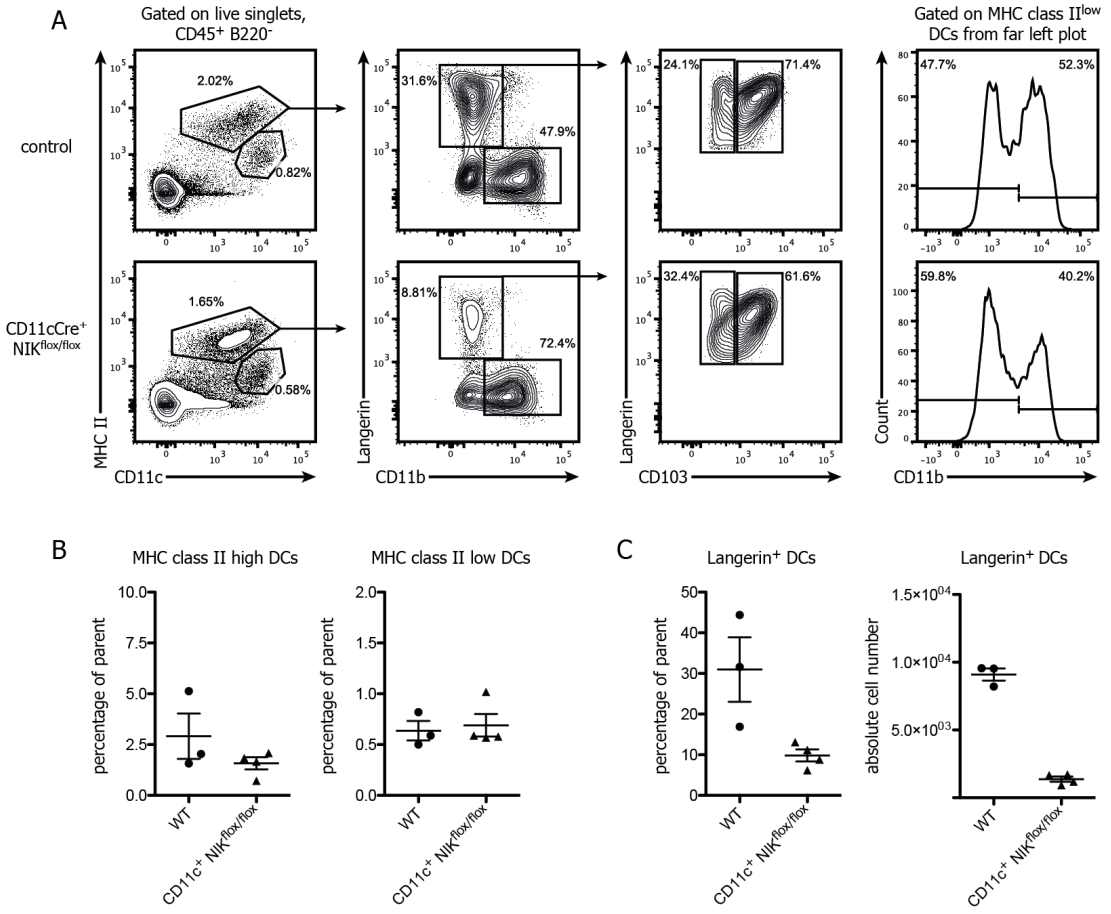

Figure 21: Selective Loss of Langerin^{+} DCs in the skin-draining lymph node of CD11c-Cre^{+} NIK$^{flox/flox}$ animals.

(A) Flow cytometric analysis of the steady state DC compartment in the skin-draining lymph node of Cre negative control (upper row) and CD11c-Cre^{+} NIK$^{flox/flox}$ (lower row) mice. Left plots have been pregated on CD45^{+} B220^{-} live cells. Far right plots indicate CD11b expression on MHC-II^{low} DCs. **(B)** Quantification of the frequency of MHC-II high and low DCs **(C)** Quantification in terms of frequency (left plot) and absolute number of Langerin^{+} CD11c^{+} MHC-II^{high} cells.

However, when we analyzed the Langerin^{+} MHC-II^{high} compartment in CD11c-Cre NIK$^{flox/flox}$ mice, we found a striking reduction both in frequency as well as absolute numbers (Figure 21a and c), that was accompanied by an increase in CD11b^{+} DCs. The proportion of CD103^{+} cells within Langerin^{+} DCs did not change.

Given that all Langerin^{+} DCs in the lymph node migrate there from the skin, we analyzed the dermal DC compartment of CD11c-Cre NIK$^{flox/flox}$ animals. Suprisingly, both the total CD11c^{+} MHC-II^{+} DC pool as well as the percentage of Langerin^{+} DCs was similar between CD11c-Cre NIK$^{flox/flox}$ and control mice (Figure 22a and b).

Thus, it seems that DC intrinsic loss of NIK signaling interferes with the migration of Langerin^{+} DCs from the skin to the lymph node. However, further experimentation is needed to investigate the underlying mechanism.

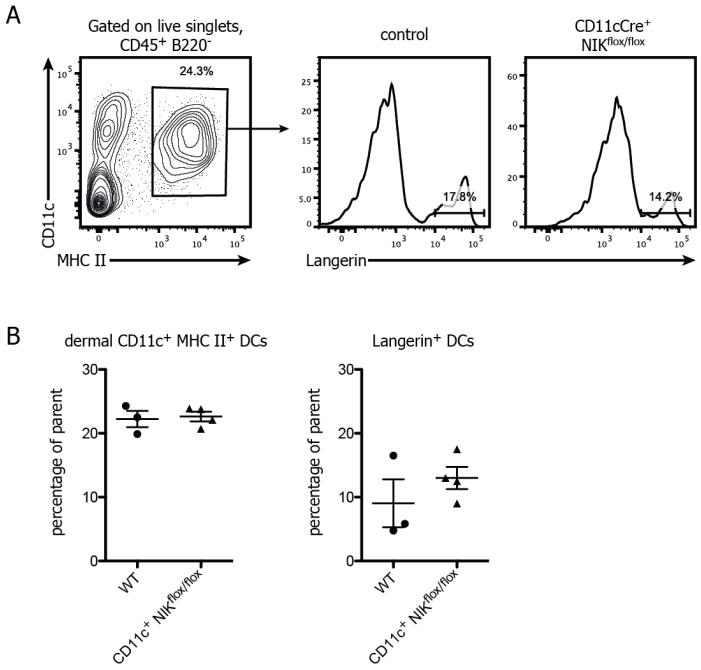

Figure 22: Langerin^{+} DCs are present at normal numbers in the dermis of CD11c-Cre^{+} NIK$^{flox/flox}$ mice.

(A) Lymphocytes were isolated from the dermis for flow cytometric analysis and pregated on CD45^{+} B220^{-} live cells. Middle and right plot show the frequency of Langerin^{+} DCs. **(B)** Quantification of the percentage of total DCs (left) and Langerin^{+} DCs (right).

DC-SPECIFIC DELETION OF NIK LEADS TO EAE RESISTANCE

Previous work from our lab suggested, that the EAE resistance of complete NIK$^{-/-}$ animals can be attributed to a DC defect (Hofmann et al., 2011). However, contradictory results have been reported (Jin et al., 2009). Therefore, we wanted to revisit the role of NIK in EAE development using our novel conditional mouse model. EAE was induced by subcutaneous immunization with MOG/CFA in CD11c-Cre NIK$^{flox/flox}$ and control animals.

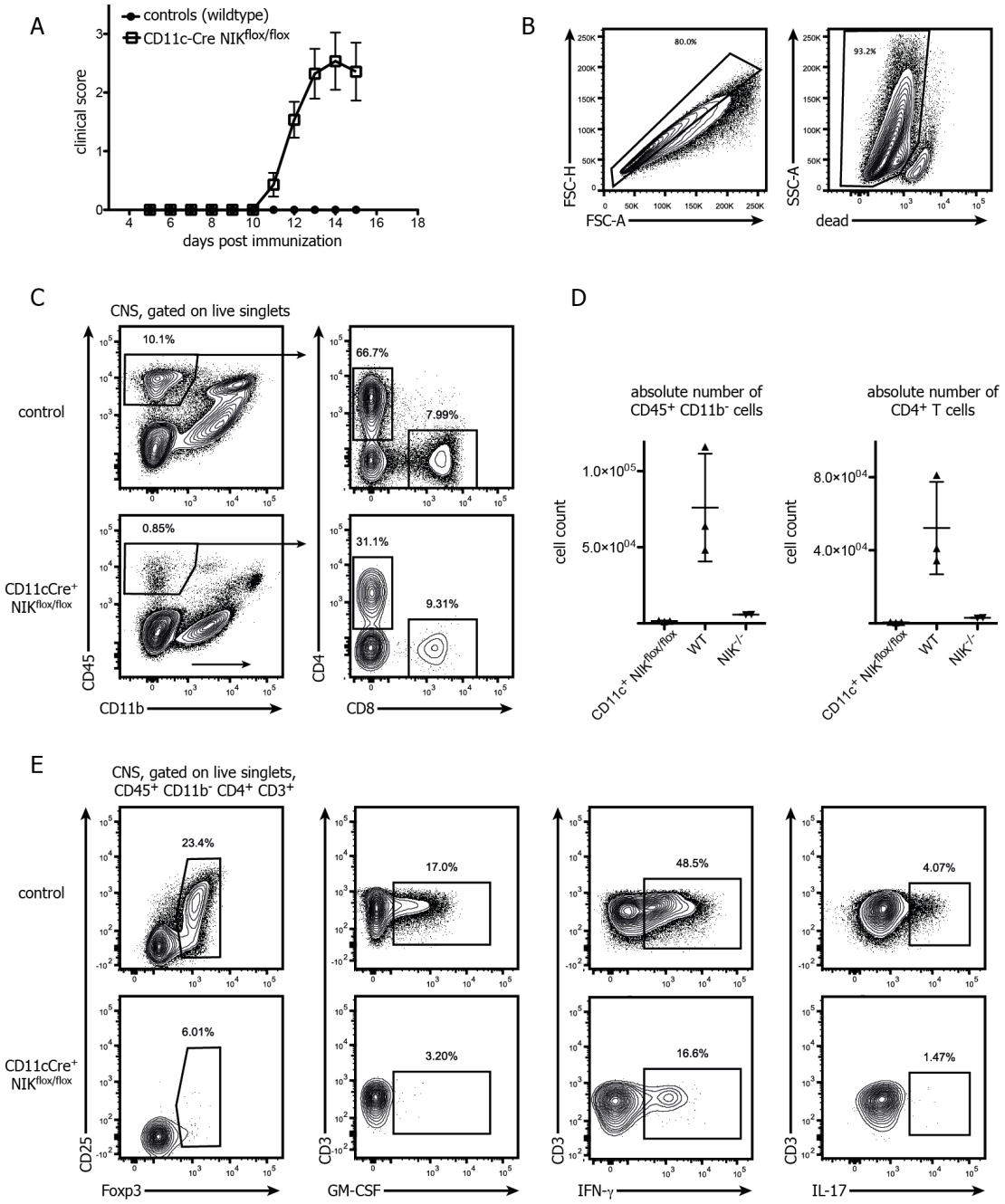

Figure 23: CD11c-Cre$^+$ NIK$^{flox/flox}$ animals are resistant to the development of EAE.

(A) CD11-Cre$^+$ NIK$^{flox/flox}$ (squares) and control B6 mice (circles) were immunized with MOG/CFA and monitored for their disease score. **(B)** Gating strategy used for the analysis of CNS infiltrating lymphocytes. **(C)** Flow cytometric analysis of the CNS infiltrates in control animals (upper panel) and CD11-Cre$^+$ NIK$^{flox/flox}$ on day 15 post immunization. **(D)** Quantification of total CD45$^+$ CD11b$^-$ infiltrating cells and CD4$^+$ T cells. **(E)** Flow cytometric analysis of the percentage of Foxp3$^+$ Tregs (left plots) and GM-CSF, IFN-γ and IL-17 production within CNS infiltrating CD4$^+$ T cells.

While the controls developed normal paralysis, no clinical signs of disease were observed in CD11c-Cre NIK$^{flox/flox}$ mice (Figure 23a). Analysis of CNS infiltrating cells on day 15 post immunization showed very few infiltrating T cells (Figure 23c and d), and within these cells the expression of pro-inflammatory cytokines remained much lower than in control mice. Also, very few FoxP3^{+} Tregs were detected (Figure 23e).
For subcutaneous antigens such as during MOG/CFA immunization, skin draining DCs play an important role for priming of antigen specific T cells. Thus, we wanted to verify whether the deficiency of Langerin^{+} DCs in the lymph node that we observed in steady state CD11c-Cre NIK$^{flox/flox}$ animals also remained in inflammatory conditions. To this end, we analyzed the inguinal and axillary lymph nodes for the presence of different DC subsets on day 15 post immunization.

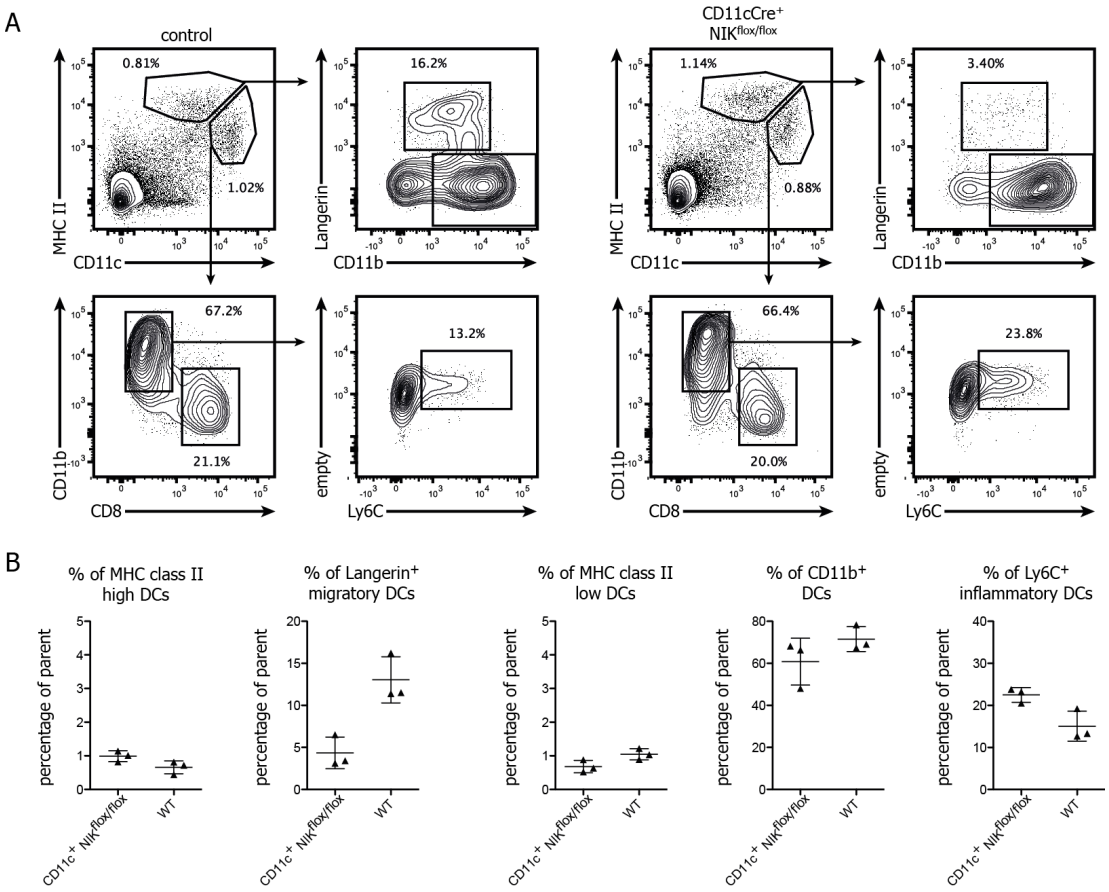

Figure 24: Under inflammatory conditions, Langerin^{+} DCs in the skin draining LN are reduced in CD11c-Cre^{+} NIK$^{flox/flox}$ animals.

(A) Flow cytometric analysis of the DC populations in the skin draining LN of control (left panel) and CD11c-Cre^{+} NIK$^{flox/flox}$ mice on day 15 post immunization with MOG/CFA. Plots have been pregated on CD45^{+} live singlets. Langerin^{+} MHC class II^{high} DCs represent skin-draining Langerhans cells and Langerin^{+} dermal DCs, Ly6^{+} CD11b^{+} MHC class II^{low} DCs represent monocyte derived inflammatory DCs. **(B)** Quantification in frequency of parent of the indicated DC subsets.

Indeed, the number of MHC-II^{high} Langerin^{+} DCs was again strongly reduced in CD11c-Cre NIK$^{flox/flox}$ lymph nodes, while the other DC subsets including cDCs were present in numbers comparable to controls (Figure 24a and b). For Ly6C^{+} inflammatory DCs there was a slight, but non-significant trend towards higher proportions.

T CELL-SPECIFIC DELETION OF NIK

Despite our previous work arguing for a critical of NIK in the context of EAE solely in DCs (Hofmann et al., 2011), we wanted to analyze whether T cell specific deletion of NIK would also influence the disease progression. For this, we immunized CD4-Cre NIK$^{flox/flox}$ mice with MOG/CFA. As shown before (Figure 18), NIK is efficiently deleted from the $\alpha\beta$ T cell compartment of CD4-Cre NIK$^{flox/flox}$ mice.

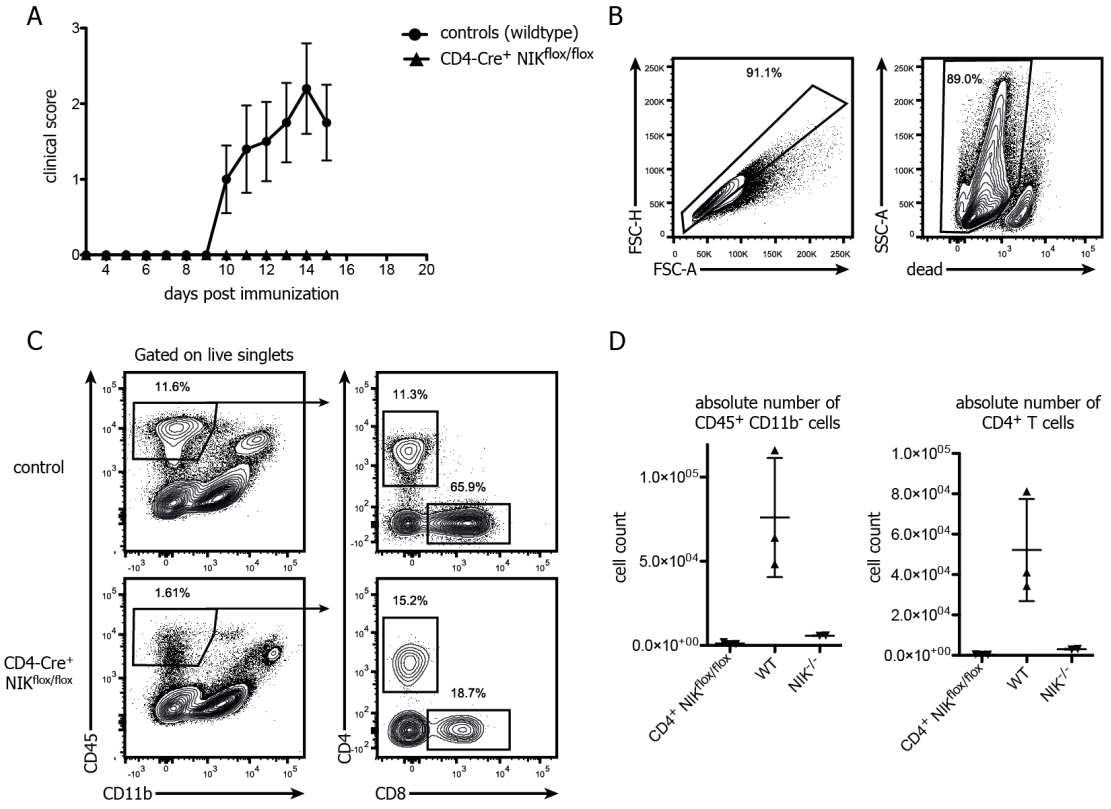

Figure 25: CD4-Cre^{+} NIK$^{flox/flox}$ animals are resistant to the development of EAE.

(A) CD4-Cre^{+} NIK$^{flox/flox}$ (triangles) and control B6 mice (circles) were immunized with MOG/CFA and monitored for their disease score. **(B)** Gating strategy used for the analysis of CNS infiltrating lymphocytes. **(C)** Flow cytometric analysis of the CNS infiltrates in control animals (upper panel) and CD4-Cre^{+} NIK$^{flox/flox}$ on day 15 post immunization. **(D)** Quantification of total CD45^{+} CD11b^{-} infiltrating cells and CD4^{+} T cells.

Surprisingly, T cell specific deletion of NIK also led to a complete lack of clinical EAE symptoms (Figure 25a). In the CNS of CD4-Cre $NIK^{flox/flox}$ mice on day 15 post immunization we could not detect any inflammatory infiltrates, and very few $CD4^+$ T cells were present (Figure 25c and d). Preliminary analysis of the cytokine production by $CD4^+$ T cells in CD4-Cre $NIK^{flox/flox}$ mice showed major impairments (data not shown). This suggests, that contrary to our previous hypotheses (Hofmann et al., 2011), T cell intrinsic NIK signaling does play a role for the induction of cell-mediated immunity. Hence, further work is needed to analyze the T cell intrinsic role of NIK in more detail in this novel conditional model.

DISCUSSION

During the past 20 years the NFκB family of transcription factors has been established without doubt as one of the most important regulators of the innate and adaptive immune system (Li and Verma, 2002; Pasparakis, 2009; Vallabhapurapu and Karin, 2009). However, the dazzling complexity of signaling pathways converging to one particular NFκB dimer has made it difficult to study and understand the functions of NFκB in straight knockout models, which also often are embryonically lethal (Doi et al., 1997). Therefore, immunologists have turned to studying individual receptors that lead to NFκB activation, as well as the molecules relaying signals from cell surface receptors to NFκB.

Among these, non-canonical NFκB signaling via the NFκB-inducing kinase (NIK) has turned out to be one of the most interesting pathways. Initially, its primary role was seen in the development of secondary lymphoid tissues (Shinkura et al., 1999; Yin et al., 2001), but during the past decade reports from many groups have established that NIK signaling is essential in many compartments of the immune system. Within the scope of this discussion, we will focus on the role of NIK in cell-mediated immunity and EAE as a model of autoimmunity, and in the development and function of γδ T cells. In the end, we will also discuss the role of NIK in the development of thymic stromal cells and open questions regarding T cell tolerance.

NIK SIGNALING AND γδ T CELL FUNCTION

NIK AND DETC DEVELOPMENT

Dendritic epidermal T cells, despite being present only in the murine epidermis, have emerged as an interesting model population to study the mechanism underlying development and function of tissue resident γδ T cell subsets. Several aspects of the molecular network governing DETC development have been unraveled recently, in particular the fact that the development of thymic Vγ5$^+$ DETC precursors depends on expression of Skint-1 by thymic epithelial cells (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006). Although Skint-1 does not seem to be a ligand for the canonical Vγ5Vδ1 TCR, it induces a signaling cascade leading to expression of DETC marker genes such as Lymphotactin (Xcl1), but also T-bet, which from studies in αβ T cells is known to induce a transcriptional program enabling IFN-γ production. In the absence of Skint-1 signals, Vγ5$^+$ thymocytes fail to upregulate T-bet, but instead start transcription of RORγt which enables these DETC precursors for production of IL-17 (Turchinovich and Hayday, 2011).

We found a similar functional switch in $V\gamma5^+$ DETC precursors isolated from $NIK^{-/-}$ thymi, posing the obvious question as to whether NIK deficient mTECs lack expression of Skint-1. Supporting this hypothesis, in preliminary experiments we found reduced Skint-1 expression in $NIK^{-/-}$ mTECs, which could explain the developmental block of $V\gamma5^+$ thymocytes. However, is NIK involved in signaling within the $\gamma\delta$ T cell compartment, or within mTECs, or both? Very recently, the group of Graham Anderson reported data supporting a model in which $V\gamma5^+$ precursors interact via RANK-RANKL with immature thymic epithelial cells, thereby inducing their functional maturation towards $Aire^+$ mTECs, which in turn via Skint-1 will stimulate the development of $V\gamma5^+$ thymocytes towards mature DETCs (Roberts et al., 2012).

This convincing and simple model would provide a compelling explanation for the DETC phenotype observed in $NIK^{-/-}$ mice, since RANK signals via NIK (Darnay et al., 1999). However, in $RANK^{-/-}$ mice (also known as TNFRSF11a or CD265) (Roberts et al., 2012), the number of DETCs in the neonatal epidermis was only reduced, while in $NIK^{-/-}$ animals we could detect almost no DETCs. This suggests that in the absence of RANK another surface receptor, probably LTβ-receptor, generates sufficient signal to generate a partial DETC population. Of note, the majority of DETCs found in the adult epidermis of $RANK^{-/-}$ animals retain expression of $V\gamma5^+$ (Roberts et al., 2012), while in $NIK^{-/-}$ animals on average 50% of the $\gamma\delta$ T cells present in the epidermis express another $\gamma\delta$ TCR specificity, that we were unable to identify so far.

Most importantly, up to date no genetic models have been employed to clarify whether RANK or LTβR signals are truly only necessary in the mTEC compartment. Several lines of evidence hint towards this model (Akiyama et al., 2008; Hikosaka et al., 2008; Roberts et al., 2012), but we wanted to use the novel conditional NIK mouse line to validate this hypothesis, and found surprising results. Despite an almost complete absence of $EpCAM^+$ medullary regions in adult CCL19-Cre $NIK^{flox/flox}$ thymi (personal communication, Lucas Onder), we could detect neither a reduction nor any phenotypical change in the epidermal DETC compartment.

This would suggest that NIK signaling in mTECs is dispensable for the formation of a normal DETC pool, but there are several explanations for this unexpected finding: first, it could be possible that instead of the reported role in the stromal compartment, $\gamma\delta$ T cell intrinsic NIK signaling is essential. To test this hypothesis, we are currently in the process of generating Lck-Cre $NIK^{flox/flox}$ mice, in which both $\alpha\beta$ and $\gamma\delta$ T cells will be devoid of NIK (unfortunately, no $\gamma\delta$ T cell specific Cre line is available yet). Second, it could be possible that the CCL19-Cre transgene does not target immature embryonic mTECs, and that NIK signaling in these early cells is

sufficient to drive DETC development. Therefore, we are planning to breed a Keratin5-Cre line (Tarutani et al., 1997) with $NIK^{flox/flox}$ animals, which should target already the earliest mTEC precursors (Rodewald, 2008).

THE ROLE OF NIK IN CYTOKINE SECRETION BY γδ T CELLS

In addition to the impaired DETC development, we have observed that the lack of NIK-signaling specifically impairs IL-17 production by mature lymphoid and tissue-resident γδ T cells, while their ability to secrete IFN-γ remains intact. This is particularly intriguing since for the majority of γδ T cells, their functional profile is set during thymic development, seemingly in dependence of self-antigen encounter (Jensen et al., 2008; Ribot et al., 2009). Moreover, the development of IL-17 producing γδ T cells seems to be supported by the thymic microenvironment only within a small developmental window early in ontogeny (Haas et al., 2012; Prinz et al., 2013). A prototypic population exemplifying this concept are $V\gamma6^+$ γδ T cells, that in ontogeny develop immediately after $V\gamma5^+$ DETCs, and are among the most potent producers of IL-17 of all γδ T cells (Shibata et al., 2008). Also, $V\gamma4^+$ γδ T cells, that can be found both in the spleen and the dermis, contain a major fraction of IL-17 producing γδ T cells (Carding and Egan, 2002).

However, when we analyzed embryonic $V\gamma4^+$ thymocytes for their ability to produce IL-17, we could not observe any impairment in $NIK^{-/-}$ thymocytes compared to their controls. The same was true for $V\gamma6^+$ embryonic thymocytes (data not shown). This is perplexing, as adult splenic $V\gamma4^+$ γδ T cells were almost devoid of IL-17 producing cells, and even *in vitro* culture with agonistic TCR stimulation was not able to evoke significant IL-17 production. One possible explanation for that could be that IL-17 producing γδ precursors start developing in the thymus, but are not able to complete their maturation program and either die by apoptosis or undergo functional re-programming, similar to our experimental observations for NIK-deficient DETCs. Yet, preliminary time-course experiments in $NIK^{-/-}$ animals show that $V\gamma4^+$ thymocytes on embryonic day 17, day 19 as well as 6 days after birth are capable of IL-17 production similar to heterozygous controls. So far we have not analyzed the peripheral γδ T cell compartment in young animals after birth, to see at which stage their ability for IL-17 production becomes lost.

Notably, RelB has already previously been described as a factor conferring the ability of IL-17 production to γδ T cells (Powolny-Budnicka et al., 2011), corroborating the observations that we have made using $NIK^{-/-}$ mice. Nevertheless, while the report from the group of Falk Weih presents solid data for a model in which γδ T cell intrinsic LTβ-receptor signaling confers full functionality to γδ T cells, it was published at a time when it was not known yet that only the embryonic or newborn

thymus can mediate $\gamma\delta_{17}$ T cell development (Haas et al., 2012). Therefore, the precise contribution of the NIK-RelB axis to the specialized thymic microenvironment during early ontogeny has not been clarified completely, particularly to which extend stromal cell types might influence this process. In this context, our novel conditional NIK mouse model will be extremely helpful in order to remove NIK signaling specifically only in T cells or in mTECS.

In addition, two very fascinating mysteries of $\gamma\delta$ T cell development remain unsolved: why do particular TCR specificities home only to particular tissues? And what mechanism selects these specificities (Vantourout and Hayday, 2013)? For $V\gamma5^+$ DETCs the molecule Skint-1 has provided an answer, but no such molecule has been identified neither for $V\gamma4^+$ dermis-homing nor for $V\gamma6^+$ lung-homing $\gamma\delta$ T cells. Interestingly, a recent study, in which different $\gamma\delta$ thymocytes based on their TCRγ chain expression were transcriptionally profiled, suggested that there are three distinct gene-expression programs: $V\gamma4^+/V\gamma6^+$ IL-17 producing $\gamma\delta$ T cells, $V\gamma5^+$ DETCs and $V\gamma1^+$/ $V\gamma7^+$ $\gamma\delta$ T cells (Narayan et al., 2012). This tripartite separation suggests that other instructive clues beside Skint-1 might exist. Given that the absence of NIK specifically impairs only some functional aspects in $\gamma\delta$ T cells it might be possible, that conditional deletion of NIK will yield further insights into the mechanism underlying the induction of these transcriptional programs.

NIK AND CELL-MEDIATED IMMUNITY

Studying the molecular pathways that govern the induction of cell-mediated immunity (CMI) enables us not only to understand immune responses against pathogenic microorganisms, but also the underpinnings of pathologic autoimmune responses.

Within the past years, a major focus of our lab has been the understanding of non-canonical NFκB signaling via NIK in the context of autoimmune neuro-inflammation. We could previously show that induction of autoreactive T cell responses proceeds unrelated to the presence or absence of secondary lymphoid tissues (Greter et al., 2009), and instead NIK signaling plays a mandatory role in dendritic cells for the induction of EAE (Hofmann et al., 2011). However, there have been contradictory interpretations, with some groups claiming T cell intrinsic impairment of T_H17 differentiation in the absence of NIK (Jin et al., 2009). Given the complicated models used previously (Hofmann et al., 2011), the only way to irrevocably answer this controversy is a genetic model. The conditional $NIK^{flox/flox}$ strain that we have described in this thesis will be the indispensable model for this.

T CELL SPECIFIC DELETION OF NIK

The results obtained so far point towards an essential role of NIK signaling in both compartments, αβ T cells and DCs. CD4-Cre $NIK^{flox/flox}$ animals were fully resistant to EAE development, but within the available time we were not able to characterize this mouse strain in more detail yet. Preliminary data suggest that T cells isolated from CD4-Cre $NIK^{flox/flox}$ mice fail to acquire their full functional potential after activation, with reduced levels of pro-inflammatory cytokines being produced, and as a result they fail to migrate into the CNS. This is similar to T cells isolated from full NIK knockout mice, but how does this reconcile with our previous findings, that $NIK^{-/-}$ T cells can be fully functional in the presence of NIK-sufficient DCs (Hofmann et al., 2011)? The used model system of a DC-specific inducible NIK allele (Sasaki et al., 2008) bred onto a $NIK^{-/-}$ background (DC^{NIK}-$NIK^{-/-}$ animals) was inherently complex, and it could be that overexpression of NIK in DCs induced some mechanism that led to T cell hyper-reactivity. Furthermore, it has been reported that CD11c-Cre can cause recombination within a small fraction of T cells, thus leading to a NIK-sufficient T cell pool. The conditional CD4-Cre $NIK^{flox/flox}$ will allow investigating these uncertainties in more detail.

Furthermore, we are currently breeding CD4-CreERt2 $NIK^{flox/flox}$ mice, in which Cre activity can be induced in $CD4^+$ T cells in a time-specific manner by the administration of Tamoxifen (Anna Sledzinska, PLOS Biol 2013 in press). In these animals we will be able to delete NIK only in mature αβ T cells but not in developing thymocytes, which will help to dissect the roles of NFκB during T cell development (Siebenlist et al., 2005) from its function in mature T cells.

It has also been reported before that NIK signaling might have differential roles in naïve and memory αβ T cells (Ishimaru et al., 2006; Rowe et al., 2013), and that in the absence of NIK the frequency and function of $FoxP3^+$ Tregs is impaired (Lu et al., 2005). We have observed the same Treg phenotype in our own studies (Hofmann et al., 2011). Again, it will be interesting to disconnect these phenotypes from the role of NIK in other cell types, particularly the thymic stroma, and analyze the function of different αβ T cell subsets using the CD4-Cre $NIK^{flox/flox}$ model. Furthermore, CD4-CreERt2 $NIK^{flox/flox}$ animals would be a unique tool for deletion of NIK *in vivo* after induction of a T cell response, which might help to clarify the proposed role of NIK in the memory T cell pool.

NIK SIGNALING AND DENDRITIC CELL SUBSETS

Only in the past decade it was appreciated that DCs not only consist of a homogenous $CD11c^+$ MHC class II^+ population, but rather of distinct subsets with specific transcriptional programs (Merad et al., 2013). To study the function of these

subsets in detail, there is a tremendous need for tools and molecules to target only single DC subsets. Not many of these have been defined yet, except two examples: Loss of BATF3 (also known as basic leucine zipper transcription factor ATF-like 3) causes a selective absence of $CD8^+$ and $CD103^+$ cDCs (Hildner et al., 2008), and knockout of the helix-loop-helix transcription factor E2-2/Tcf4 causes the specific loss of plasmacytoid DCs (Cisse et al., 2008).

Here, we have found that DC-specific deletion of NIK causes a selective reduction of migratory $Langerin^+$ cDCs in the skin-draining lymph nodes both under steady state and inflammatory conditions. Surprisingly, neither $Langerin^+$ dermal DCs nor epidermal Langerhans cells showed any obvious phenotypical differences in CD11c-Cre^+ $NIK^{flox/flox}$ animals. Therefore, it seems that NIK signaling is either essential for the migration of skin DCs to the draining LN, or for their survival within the LN, which we have to address in further experiments.

Already in the 1990s LTα (which is upstream of NIK) has been implicated in regulating the number of DCs in peripheral lymphoid organs (Wu et al., 1999). In line with this, a DC-intrinsic requirement for LTβ-receptor signaling in maintaining the lymphoid DC population was reported (Kabashima et al., 2005) . Also, in the absence of RelB the numbers of both thymic DCs (Burkly et al., 1995) and splenic $CD8^-$ DCs (Wu et al., 1998) are strongly reduced. In line with this finding, the knockout of TRAF-6 also causes a loss of $CD8^-$ splenic DCs (Kobayashi et al., 2003). Since both TRAF-6 and RelB are directly upstream and downstream of NIK, respectively, it is surprising that in $NIK^{aly/aly}$ mice the frequencies of $CD8^+$ and $CD8^-$ DCs in the spleen were found to be comparable to controls (Lind et al., 2008). However, all the above-mentioned knockouts have several impairments in stromal cell function and hence splenic structure, which in turn again might influence the DC pool. Furthermore, the contribution of LTβ–receptor signaling via NIK in non-lymphoid tissue DCs has not been investigated yet. The CD11c-Cre^+ $NIK^{flox/flox}$ mice will provide a unique tool to address these open questions.

In the context of EAE, the absence of skin-draining DCs in the lymph node would provide a simple and plausible explanation for the EAE resistance observed in CD11c-Cre^+ $NIK^{flox/flox}$ mice, since it has been reported that after depletion of $Langerin^+$ $CD103^+$ DCs, subcutaneous immunization with MOG/CFA causes only mild EAE with a delayed disease onset (King et al., 2010). This notion has been challenged by a subsequent report using a genetic model, namely $Batf3^{-/-}$ mice that specifically lack all $CD11b^-$ DCs. Despite the complete loss of dermal $CD103^+$ DCs, these mice developed normal EAE (Edelson et al., 2011). Given this finding, the reason for the EAE resistance in CD11c-Cre^+ $NIK^{flox/flox}$ mice must be more complex.

In line with our published data on NIK$^{-/-}$ DCs (Hofmann et al., 2011) and previous work by others (Lind et al., 2008), we found reduced production of IL12/23p40 by DCs isolated from CD11c-Cre^{+} NIK$^{flox/flox}$ mice. As a next step, it would be interesting to dissect whether there is a selective effect on either secretion of IL-12 or IL-23, as the latter is absolutely essential for the induction of pathogenic, autoreactive T cells (Cua et al., 2003). Furthermore, we plan to analyze the ability for production of other pro-inflammatory cytokines such as IL-6.

Notably, it has already been reported before that NIK$^{aly/aly}$ splenic DCs are impaired in their ability for cross-priming (Lind et al., 2008). In the same study, gene profiling of splenic NIK$^{aly/aly}$ DCs revealed that after *in vivo* stimulation with anti-CD40 a plethora of genes relevant to DC function failed to be upregulated, including IL-6, CD86 and CD274 (Programmed Death Ligand-1). However, by now it is well established that DCs differ in their function depending on their anatomic location, and obviously it is inherently impossible to study LN-resident DCs in NIK$^{aly/aly}$ or NIK$^{-/-}$ animals because of the complete lack of LNs. Hence, to expand previous findings on DC function (Lind et al., 2008), we plan to analyze the effects of DC-specific deletion of NIK in-depth in different DC subsets both in lymphoid and non-lymphoid tissues.

NIK SIGNALING AND THE ESTABLISHMENT OF CENTRAL TOLERANCE

As discussed above and in the introduction, T cell function has extensively been studied in NIK$^{aly/aly}$ and NIK$^{-/-}$ mice, with varying experimental interpretations (Ishimaru et al., 2006; Lu et al., 2005; Matsumoto et al., 2002; Rowe et al., 2013). One major caveat of some of these studies is that the influence of abolished NIK signaling on the development of thymic epithelial cells, and thus on T cell development, was ignored. It has been shown without doubt that NIK$^{aly/aly}$ mice, similar to TRAF-6$^{-/-}$ and RelB$^{-/-}$ animals do not form a structurally normal thymic medulla, with decreased numbers of mature mTECs (Akiyama, 2005; Burkly et al., 1995; Kajiura et al., 2004). Promiscuous gene expression of tissue restricted self-antigens, which is executed by mTECs under control of the Autoimmune regulator (Aire) (Mathis and Benoist, 2009), is an essential tolerance mechanism both for purging the developing T cell repertoire of self-reactive specificities and for the induction of Tregs (Anderson et al., 2002; Aschenbrenner et al., 2007). Furthermore, it has been postulated that a small population of so-called natural T_H17 cells is selected in the thymus based on interaction with MHC class II on mTECs, similar to agonist induced selection of Tregs (Kim et al., 2011). Also, previously published data from our lab support a model in which thymic DCs in the absence of NIK influence

the developing T cell pool in terms of its effector function (Hofmann et al., 2011). Therefore, it is inherently difficult to disconnect any T cell phenotype from the thymic impairments seen in straight $NIK^{-/-}$ animals, even with the use of bone marrow chimeric approaches.

The conditional $NIK^{flox/flox}$ allele will for the first time allow studying the role of NIK signaling in the T cell compartment and in mTEC development and central tolerance induction separately. In collaboration with the Institute of Immunobiology in St. Gallen we have crossed CCL19-Cre $NIK^{flox/flox}$ animals, in which Cre will be active in stromal cells of the lymph nodes and mTECs (Onder et al., 2013). According to preliminary data, in CCL19-Cre $NIK^{flox/flox}$ thymi NIK is specifically deleted in mature MHC-II^{high} mTECs. As a result, the structure of the thymic medulla is almost completely eliminated, and instead a population of immature $CD205^{+}$ $EpCam^{+}$ mTECs accumulates (data generated by Lucas Onder, Figure 26). While it has already been reported before that signaling events from the LTβ-receptor and RANK are important for the formation of the mTEC network (Boehm et al., 2003; Hikosaka et al., 2008; Rossi et al., 2007), this mouse model will allow to investigate the development of the thymic stroma, and the resulting impact on the developing T cell pool in much more detail than previously possible.

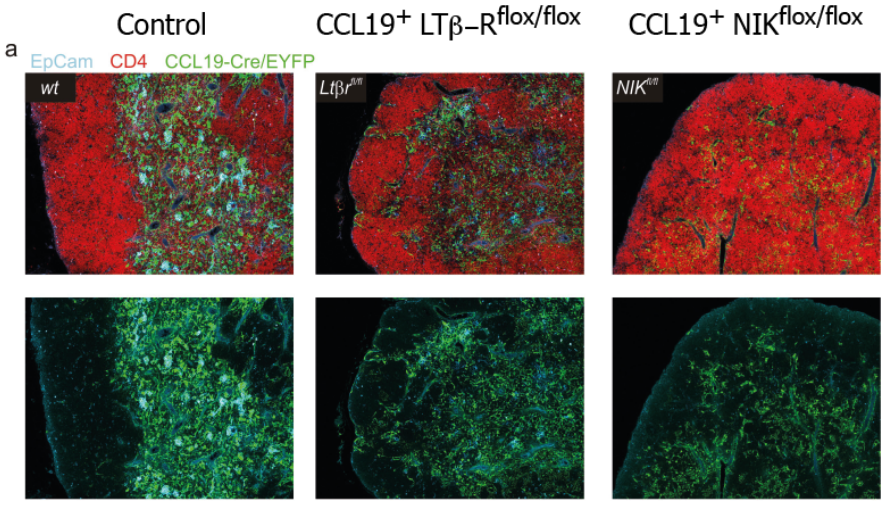

Figure 26: Deletion of NIK in mTECs causes loss of the thymic medulla

Microscopic analysis of the thymi of adult control (left panel), CCL19-Cre^{+} LTβ–$R^{flox/flox}$ (middle panel) and CCL19-Cre^{+} $NIK^{flox/flox}$ animals. Green represents CCL19-YFP, red staining for CD4, and blue staining for EpCam (marking mTECs). Pictures and data have been generated by Lucas Onder, Cantonal Hospital of St. Gallen.

Beside mTECs, thymic DCs are essential for deleting auto-reactive thymocytes (Gallegos, 2004), and also the induction of Tregs (Proietto et al., 2008). Previous work in our lab suggests that in the absence of NIK signaling, thymic DCs are impaired in their functional capacity, and they produce less of the chemokines needed for the guided migration of developing thymocytes (Hofmann et al., 2011). Again, it has not been clarified, if these phenotypes are caused by impaired DC-intrinsic NIK signaling or by the inherent structural defects in complete $NIK^{-/-}$ mice. Hence, it will be interesting to investigate whether CD11c-Cre^{+} $NIK^{flox/flox}$ mice show any defects in thymic DC function and T cell development, which for time reasons we were unable to analyze yet.

NIK SIGNALING – NON-CANONICAL ONLY?

The notion of two separate signaling entities driving the activation of distinct NFκB-dimers has been widely accepted since its initial discovery (Dejardin et al., 2002; Malinin et al., 1997). However, there is accumulating evidence that this strict separation does not always reflect reality, as some reports show that NIK can signal into the classical pathway. First, depending on the upstream receptor engaged, NIK seems to engage either the classical or the non-canonical pathway (Ramakrishnan et al., 2004). Also, it has been suggested that NIK is able to "amplify" classical NFκB activation (Zarnegar et al., 2008).

Along these lines, preliminary data from our lab indicate that the downstream target of NIK might not necessarily be only NFκB2 (Janin Hofmann, personal communication): Lethally irradiated wild-type animals that were reconstituted with $RelB^{-/-}$ bone marrow (hence having a RelB deficient hematopoietic compartment) were fully susceptible towards the development of EAE, which is the opposite of the phenotype observed in wild-type chimeras reconstituted with $NIK^{-/-}$ bone marrow. Also, $NF\kappa B2^{-/-}$ animals have been reported to have normal numbers of $FoxP3^{+}$ Tregs in the periphery (Zhu et al., 2006), while our own data show that both $NIK^{aly/aly}$ and $NIK^{-/-}$ mice have a 50% reduction in the frequency of Tregs (Hofmann et al., 2011). Finally, very recent work implementing mathematical modeling of signaling pathways suggests, that RelB can be a target of classical NFκB signaling (Shih et al., 2012).

Clearly, further biochemical studies are needed to understand the intracellular signaling network of NFκB activation in more detail, but it can be said without doubt that signals relayed via NIK play distinct roles in the activation of various immune cells.

CONCLUDING REMARKS

For a long time, NIK deficient mice have served as an animal model for studying immune responses in the absence of lymph nodes. Only within the past decade it became evident that loss of NIK leads to cell-intrinsic defects in various immune compartments, including B cells, T cells, DCs and stromal cells. Previous work from our lab has identified NIK as an essential signaling entity for the development of EAE, irrespective of the lack of lymph nodes in $NIK^{-/-}$ animals, thus emphasizing the need to understand the function of NIK particularly in T cells and DCs.

Within this thesis, I have identified additional functions for NIK in the γδ T cell compartment: First, NIK signaling is essential to provide γδ T cells with the ability to produce IL-17. Second, in the absence of NIK, the epidermal DETC pool cannot form normally. The precise underlying mechanism seems to be centered on the function of NIK in thymic epithelial cells, but further work is needed to delineate the series of events that allow for normal development of γδ T cells.

Furthermore, I have described a novel conditional mouse model, that for the first time allows the cell-type specific deletion of NIK without confounding effects from the structural deficits in complete knockout models.
In line with previous data from our lab, DC-specific deletion of NIK causes mice to be resistant towards the development of EAE. Furthermore, NIK signaling seems to play very specific roles for the normal migration and function of DCs in the lymph node. Additional experiments using CD11c-Cre $NIK^{flox/flox}$ mice will yield further insight into the function of NIK signaling in DCs beyond its known role in relaying CD40-signals.

In summary, the conditional NIK mouse line described here will be very useful to add further insight into the role of NIK signaling within the immune system.

MATERIALS AND EXPERIMENTAL PROCEDURES

MICE AND ANIMAL EXPERIMENTS

All mice were kept in-house in individually ventilated cages under specific pathogen-free (SPF) conditions. C57BL/6 (wild-type) mice were purchased from Janvier. $NIK^{flox/wt}$ mice were generated by Taconic-Artemis (Cologne, Germany) and maintained in our facility. CD11c-Cre mice were generously provided by Boris Reizis (Columbia University, New York, USA), and CD11c-Cre-GFP mice were obtained from Alexander Chervonsky (Jackson Laboratory, Ben Harbor, USA). CD4-Cre mice were obtained from Jackson Laboratories. CCL19-Cre mice were generated by Lucas Onder (Chai et al., 2013) and crossed with $NIK^{flox/flox}$ mice at the Cantonal Hospital St. Gallen.

To obtain embryos of a specifc age, timed matings were set up: male and female mice were mated over night, and females were observed for signs of pregnancy. Pregnant females were euthanized by CO_2 inhalation, embryos were removed and euthanised by decapitation.

Experimental autoimmune encephalomyelitis (EAE) was induced as described previously (Gyülvészi et al., 2009). Briefly, mice were immunized subcutaneously with 200 µg of MOG_{35-55} peptide (MEVGWYRSPFS-RVVHLYRNGK; GenScript) emulsified in CFA (Difco) in a total volume of 200 µl and two intraperitoneal injections of 200 ng Pertussis toxin (Sigma) on day 0 and 2. For passive EAE experiments, spleen and lymph node cells were harvested from donor mice of the indicated genotype on day 7 post immunisation, restimulated *in vitro* for two days with 20 µg/ml MOG and 10 ng/ml IL-23 and then i.v. transferred to sublethally (550 Rad) irradiated WT or $Rag1^{-/-}$ recipients.

All animal experiments were approved by local authorities (Kantonales Veterinäramt, Zurich) and performed under the appropriate experimental license (55-2009 and 86-2012).

DNA ISOLATION AND GENOTYPING

Biopsies were obtained either by ear-punching adult mice, or by toe-clipping pups before reaching an age of 12 days. Biopsies were incubated in 500 µl tail lysis buffer containing 10 µl Proteinase K for 2-6 hours at 55°C, followed by centrifugation for 5 minutes at 13.000 rpm to pellet undigested tissue. The supernatant was transferred to a fresh tube and 500 µl of Isopropanol were added to precipitate the DNA, followed by centrifugation for 20 minutes at 13.000 rpm / 4°C. The supernatant was discarded, the pellet was washed with 500 µl of 70% Ethanol and centrifuged again

for 5 minutes at 13.000 rpm / 4°C. After removal of the supernatant, the DNA pellets were air-dried for 10-15 minutes and then resuspended in 50-100 µl of TE buffer. If the DNA did not fully dissolve, samples were incubated for 10-15 minutes at 55°C.

For genotyping, 1 µl of the purified DNA was used as template, together with the following primers and PCR protocols. PCR products were analyzed by running a standard 1-2% agarose Gel in TAE buffer.

NIK-knockout:

10x buffer containing Mg^{2+} (New England Biolabs):	3 µl
dNTPs (10 mM)	1 µl
5 NIK primer (AGT CCA ATT CCA TGT TGC TGC TGT)	1 µl
3 Neo primer (ATC TTG TTC AAT GGC CGA TCC CAT)	1 µl
3 NIK primer (TCT GAG ATA GGC ATA TCC CTG GCT)	1 µl
DNA template	1 µl
TAQ polymerase (New England Biolabs)	0.2 µl
PCR water	21.8µl

Denaturation:	94 °C	4 minutes
Elongation:	94 °C	30 sec
	60 °C	30 sec
	72 °C	30 sec
	repeat 35 times	
Final elongation:	72 °C	5 minutes
Hold:	10 °C	

Endogenous band: 270 bp
Recombinant band: 500 bp

NIKflox:

10x buffer without Mg^{2+} (New England Biolabs):	2.5 µl
$MgCl_2$ (50 mM)	1 µl
dNTPs (10 mM)	0.5 µl
5 primer (TAT GAA CTG CTC CCG TTT CG)	0.5 µl
3 primer (CCT GTG CAT CAC AGA GTA TAC TAG C)	0.5 µl
DNA template	1 µl
TAQ polymerase (New England Biolabs)	0.2 µl
PCR water	18.8µl

Denaturation:	94 °C	5 minutes
Elongation:	94 °C	30 sec
	60 °C	30 sec
	72 °C	60 sec
	repeat 35 times	
Final elongation:	72 °C	10 minutes
Hold:	10 °C	

Endogenous band: 357 bp
Recombinant band: 541 bp

CD11c-Cre

10x buffer without Mg^{2+} (New England Biolabs):	2.5 µl
$MgCl_2$ (50 mM)	1 µl
dNTPs (10 mM)	1 µl
5 primer (ACT TGG CAG CTG TCT CCA AG)	0.5 µl
3 primer (GCG AAC ATC TTC AGG TTC TG)	0.5 µl
DNA template	1 µl
TAQ polymerase (New England Biolabs)	0.25 µl
PCR water	17.25 µl

Denaturation: 94 °C		4 minutes
Elongation:	94 °C	30 sec
	63 °C	45 sec
	72 °C	60 sec
	repeat 35 times	
Final elongation:	72 °C	10 minutes
Hold:	10 °C	

Recombinant band: 300 bp

CD4-Cre

10x buffer without Mg^{2+} (New England Biolabs):	2.5 µl
$MgCl_2$ (50 mM)	1 µl
dNTPs (10 mM)	1 µl
5 primer (CCC AAC CAA CAA GAG CTC AAG G)	1 µl

3 primer (CCC AGA AAT GCC AGA TTA CG)		1 µl
DNA template		1 µl
TAQ polymerase (New England Biolabs)		0.2 µl
PCR water		17.3 µl

Denaturation: 95 °C		3 minutes
Elongation:	94 °C	45 sec
	63 °C	45 sec
	72 °C	60 sec
	repeat 35 times	
Final elongation:	72 °C	5 minutes
Hold:	10 °C	

Recombinant band: 600 bp

CD4-Cre ErT2

10x buffer with Mg^{2+} (New England Biolabs):	2.5 µl
dNTPs (10 mM)	1 µl
Primer 1 (TCTTAGTTTGGCAGGACCT)	1 µl
Primer 2 (CGGCATCAACGTTTTCTTTT)	1 µl
Primer 3 (TCTTCTTCTGGGAACTCTCG)	1 µl
DNA template	1 µl
TAQ polymerase (New England Biolabs)	0.25 µl
PCR water	16.5 µl

Denaturation:	95 °C	3 minutes
Elongation:	94 °C	45 sec
	63 °C	45 sec
	72 °C	45 sec
	repeat 35 times	
Final elongation:	72 °C	5 minutes
Hold:	10 °C	

Endogenous band: 400 bp
Recombinant band: 450 bp

General Cre PCR

10x buffer with Mg^{2+} (New England Biolabs):	1.2 µl
dNTPs (10 mM)	0.25 µl
Transgene forw (GCG GTC TGG CAG TAA AAA CTA TC)	1.2 µl
Transgene rev (GTG AAA CAG CAT TGC TGT CAC TT)	1.2 µl
Pos ctrl forw (CTA GGC CAC AGA ATT GAA AGA TCT)	1.2 µl
Pos ctrl rev (GTA GGT GGA AAT TCT AGC ATC ATC C)	1.2 µl
DNA template	1 µl
TAQ polymerase (New England Biolabs)	0.1 µl
PCR water	4.65 µl

Denaturation:	94 °C	3 minutes
Elongation:	94 °C	30 sec
	51.7 °C	60 sec
	72 °C	60 sec
	repeat 35 times	
Final elongation:	72 °C	3 minutes
Hold:	12 °C	

Cre Transgene: 100 bp
Internal Control: 324 bp

LYMPHOCYTE ISOLATION FROM SPLEEN, THYMUS AND LN

For isolation of bulk lymphocytes, spleen, thymus and lymph nodes were mechanically disrupted using either microscopic glass slides or a syringe plunger and a 70 µm cell strainer. For spleens, red blood cell (RBC) lysis was performed using 1 ml of RBC lysis buffer and 5-10 minutes of incubation on ice, followed by a washing step with 10 ml of PBS. Unless stated otherwise, all centrifugation steps were carried out for 5 minutes at 500 g / 4°C.

Embyonic thymi were obtained by decapitating embryos of the indicated developmental stage, followed by careful dissection under a stereo microscope (Leica). Thymi were disrupted mechanically using a syringe plunger and a 70 µm cell strainer.

For the isolation of DCs from the lymph node, Collagenase D digestion was used: lymph nodes were cut into small pieces, incubated in 2 ml of Collagenase D (0.4 mg/ml) and DNase (0.1 mg/ml) in RPMI containing 2%FCS and 25mM HEPES

for 30-45 minutes at 37°C, followed by mechanical disruption with syringes. The cell suspension was filtered though a 70 µm cell strainer and used for further procedures.

LYMPHOCYTE ISOLATION FROM CNS

Mononucleated cells were obtained from CNS tissues as described (Gyülvészi et al., 2009): mice were euthanized using CO_2 inhalation. Afterwards, animals were perfused using 25 ml of ice-cold PBS, the spinal cord was flushed out of the spine by hydrostatic pressure, and the brain was removed from the scull. Brain and spinal cord were pooled and cut into small pieces using scissors, followed by 30-45 minutes of digestion with 0.4 mg/ml Collagenase D (Roche) and 0.1 mg/ml DNase (Sigma) in IMDM containing 25mM HEPES and 2% FCS at 37°C. The digest was stopped by addition of 1:100 of 0.5M EDTA, and remaining pieces of tissue were homogenized using syringes and 20 gauge needles. After washing, the cell suspension was loaded onto a continuous 28% Percoll (GE) gradient and centrifuged for 30 minutes at 15.000 g. Myelin was removed from the upper Percoll layer, and mononuclear cells were obtained from the interphase, washed twice with PBS and used for further procedures.

LYMPHOCYTE ISOLATION FROM THE SKIN

Isolation of lymphocytes from the murine skin was adapted based on previously published protocols (Ginhoux et al., 2009). Unless stated otherwise, ears were used as skin samples. Mouse ears were split into dorsal and ventral parts using forceps and incubated at 37°C for 90-105 minutes in HBSS (Invitrogen) containing Ca^{2+}/Mg^{2+} and 2.4 mg/ml of Dispase (Sigma). Afterwards epidermal sheets were removed from the dermis, and dermis and epidermis were separately cut into small pieces using scissors, followed by incubation in HBSS containing Ca^{2+}/Mg^{2+} and 10% FBS and 0.2-0.4 mg/ml Collagenase Type IV (Sigma). The resulting suspension was homogenized using syringes and 19 gauge needles, followed by filtering through a 70 µm cell strainer and further use in downstream applications.

For embryos, after decapitation the whole body skin was obtained and treated the same way as ears.

LYMPHOCYTE ISOLATION FROM THE LUNG

For lymphocyte isolation from the lung, lungs were collected and cut into small pieces using scissors and incubated in 1 mg/ml collagenase D (Roche) and 0.1 mg/ml DNase (Sigma) in IMDM containing 25mM HEPES and 2% FCS for

approximately 60 minutes at 37°C. The digest was stopped by addition of 1:100 of 0.5M EDTA, and remaining pieces of tissue were homogenized using syringes and 20 gauge needles. The cell suspension was filtered via 70 µm cell strainers and washed with PBS. Red blood cell (RBC) lysis was performed using 1 ml of RBC lysis buffer and 5-10 minutes of incubation on ice, followed by a washing step with 10 ml of PBS. The obtained cells were used for further procedures.

ENRICHMENT OF THYMIC STROMAL CELLS

Thymi were cleaned of fat and connective tissue and cut into pieces using small scissors, incubated with collagenase/dispase (Roche, final conc. 0.4 mg/ml) including DNase (final conc. 0.1 mg/ml) in IMDM containing 25mM HEPES and 2% FCS at 37°C for 45 minutes. For each thymus, 1-2 ml of collagenase/dispase medium was used. To disrupt cell-cell interactions, the suspension was resuspended frequently. After 45 minutes the digest was stopped by addition of EDTA to yield a final concentration of 5 mM and incubation for 5 minutes at 37°C. After EDTA incubation cells were transferred to ice immediately and washed with PBS. After complete removal of the supernatant the pellet was carefully resuspended in high density percoll (GE Healthcare, ρ = 1.115, 45.2 g of PBS mixed with 5.217 g of 10x PBS). An intermediate layer of low density percoll solution (GE Healthcare, ρ = 1.045 – 1.06 according to titration, 22.3 g of high density percoll mixed with 26 g of 1x PBS) followed by an upper layer of medium was very carefully pipetted to yield an inverse discontinous density gradient.

After centrifugation at 1350 g for 30 minutes at 4°C (without brake, medium acceleration) low density cells (including mTECs and DCs) were removed from the upper interphase. Cells were washed with PBS und used for subsequent procedures.

FLOW CYTOMETRY

Flow cytometric analysis was performed following standard methods (reviewed in (McLaughlin et al., 2008; Perfetto et al., 2004)). All flourochrome-conjugated antibodies used were obtained either from BD, BioLegend or eBioscience and used according to titration experiments. Commonly used clones are listed in the table below. For all experiments, dead cells were excluded from the analysis using an Aqua Live/Dead fixable staining reagent (Invitrogen) (Perfetto et al., 2010), and doublets were excluded by FSC-Area vs FSC-Height gating.

For intracellular cytokine staining, cells were incubated 3-5 hours in IMDM containing 10% FCS with PMA (50ng/ml), Ionomycin (500ng/ml) and GolgiPlug (containing Brefeldin A, BD, 1:1000 dilution) at 37°C. Cytofix/Cytoperm (BD) was

used according to the manufacturers instructions, and Perm/Wash Buffer was either obtained from BD or prepared in-house.

Flow cytometric analysis was carried out using either a FACSCanto II (BD) or a LSR II Fortessa (special order research product, BD). Optical configurations of the used instruments are listed below. Single stained compensation controls were prepared by using anti-rat/hamster Comp Beads and the flourophores used in the particular experiment. Cell sorting was carried out using a FACSAria III (BD). Data analysis was performed using FlowJo 9.x and 10.0.0.x (TreeStar).

Optical configuration of FACSCanto II			
Laser	**Mirror**	**Filter**	**Used dyes**
Violet 405nm	502LP	510/50	AmCyan, Aqua L/D
		450/50	PacificBlue, BV421
Blue 488nm	735LP	780/60	PE-Cy7
	655LP	670LP	PerCP, PerCP-Cy5
	556LP	585/42	PE
	502LP	530/30	FITC
		488/10	SSC
Red 640nm	735LP	780/60	APC-Cy7
	685LP	660/20	APC

Optical configuration of LSR Fortessa			
Laser	**Mirror**	**Filter**	**Used dyes**
Violet 405nm	735LP	800/50	Brilliant Violet 785
	685LP	710/50	Brilliant Violet 711
	640LP	670/30	Brilliant Violet 650
	600LP	610/20	Brilliant Violet 605
	505LP	525/50	AmCyan, Aqua L/D
		450/50	PacificBlue, BV421
Blue 488nm	635LP	690/50	PerCP/PerCP-Cy5.5
	505LP	525/50	FITC
		488/10	SSC
YellowGreen 561nm	750LP	780/60	PE-Cy7
	685LP	710/50	PE-Cy5.5
	635LP	670/30	PE-Cy5
	600LP	610/20	PE-Texas Red, PE-CF594
		582/15	PE
Red 640nm	750LP	780/60	APC-Cy7
	685LP	730/45	AlexaFluor700
		670/30	APC

The antibody clones used for flow cytometry experiments were as follows:

Clone	Target	Manufacturer
17A2	CD3	eBioscience / BioLegend
GK1.5	CD4	BioLegend / BD
53-6.7	CD8	BioLegend / BD
M1/70	CD11b	BioLegend
N418	CD11c	BioLegend
M1/69	CD24	BioLegend
PC61	CD25	BD
LG.3A10	CD27	BioLegend/BD
IM7	CD44	BD
30F11	CD45	BioLegend / BD
C363-16A	CD45RB	BD
MEL-14	CD62L	BD
H1.2F3	CD69	BioLegend / BD
16-10A1	CD80	BioLegend
GL1	CD86	BioLegend
2E7	CD103	BioLegend
TM-β1	CD122	BD
RA3-6B2	B220	BD
M5/114.15.2	I-A/I-E	BioLegend
GL3	TCR γδ	BioLegend / BD
2.11	Vγ1.1	BioLegend
UC3-10A6	Vγ4	BioLegend
536	Vγ5	BioLegend
PK136	NK1-1	BD
Al-21 or HK1.4	Ly6C	BD / eBioscience
1A8	Ly6G	BD
XMG1.2	IFN-γ	BioLegend / BD
TC11-18H10 or 17B7	IL-17	BioLegend / BD
MP6-XT22	TNFα	BioLegend
MP1-22E9	GM-CSF	BD
FJK-16s	Foxp3	eBioscience
eBioL31	Langerin (CD207)	eBioscience
G8.8	EpCam	BioLegend

MACS PURIFICATION OF DCS

For bulk enrichment of splenic DCs, spleens were cut into small pieces with fine scissors and incubated in 3 ml of IMDM containing 2% FCS, 25 mM HEPES and 0.4 mg/ml Collagenase D (Roche) for 30 minutes. Remaining tissue was mechanically disrupted using a syringe plunger and a 70 µm cell strainer. RBC lysis was performed using 1 ml of RBC lysis buffer and 5-10 minutes of incubation on ice, followed by a washing step with 10 ml of PBS.

Afterwards, cells were labeled with CD11c-Microbeads (Miltenyi, clone N418) according to the manufacturers instructions and positive cells were purified using an AutoMACS (Miltenyi). The positive fraction was collected, washed with PBS and used for downstream applications.

PREPARATION OF EPIDERMAL SHEETS

For preparation of epidermal sheets, ears of the indicated genotypes were collected and split into dorsal and ventral halves using forceps. Afterwards, ears were incubated in 0.5 M Ammonium-Thiocyanate for 90 minutes at room temperature to detach the epidermis from the dermis. Epidermal sheets were carefully removed using forceps, washed in PBS and mounted on a microscopic glass slide using a fine brush. Sheets were allowed to airdry for 25 minutes at room temperature (RT), and then incubated in Aceton for 10 minutes at RT. After three consecutive washes in PBS containing 1% of Tween, the epidermal sheets were stored at 4°C or directly used for staining. For this, the samples were incubated with the respective antibodies for at least 6 hours at 4°C (CD3-Bio and Streptavidin AlexaFlour 647, or I-A^b AlexaFlour 488).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For the IL-12/23p40 ELISA the corresponding kit was obtained from BD (IL-12 OptEIA) and used in slightly modified form as follows. Capturing antibody was diluted 1:250 in coating buffer, and ELISA plates (BD) were coated with 50 µl per well at 4°C over night. Plates were washed with PBS-Tween (1%) for three times and blocked with assay diluent (PBS with 10% FBS) for 1 h at room temperature (RT). IL-12/23p40 standard (BD) and samples were diluted in assay diluent according to the experimental setup, added to the plate in duplicates and incubated for 2 hours at RT. After that, the plate was washed with PBS-Tween for five times. Biotinylated detection antibody was diluted 1:1000 in assay diluent, 50 µl per well were added and incubated for 1 hour at RT. Strepativdin-horseradish peroxidase (HRP) was diluted 1:1000 in assay diluent, and 50 µl per well were added and incubated for 30 minutes at RT. After 5-8 wash steps with PBS-Tween, 50 µl of stabilized chromogen (TMB, Invitrogen) were added. When the highest standard reached a dark-blue color, the reaction was stopped by addition of 50 µl of H_2SO_4. Absorption at 450 nm was measured using an ELISA reader.

RNA ISOLATION, cDNA GENERATION AND qPCR ANALYSIS

For RNA isolation sorted cells were pelleted and either stored at -80°C or directly processed. 1 ml of Trizol (Applichem) was added, and after thorough resuspension cells were incubated for 15 minutes at RT. 200 µl of Chloroform was added, followed by mixing. The resulting suspension was transferred to a gel-lock tube and centrifuged for 20 minutes at 13.200 g / 4°C. The aqueous (upper) phase was transferred to a fresh tube, an equal volume of cooled 70% Ethanol was added and mixed. Afterwards, RNA was isolated using the PureLink RNA Micro Kit (Invitrogen), following the manufacturers instructions. RNA was eluted in 12 µl of RNAse free water.

10 µl of the resulting RNA solution was heated to 65°C for 5 minutes, quenched on ice and used for reverse transcription with M-MLV (Invitrogen) as follows:

1 µl random primers (100 ng/µl)
1 µl dNTPs (10 mM)
4 µl First Strand buffer (5x)
2 µl DTT (0.1M)
1 ml RNAse OUT
1 µl M-MLV reverse transcriptase (200 Units/µl)
10 µl RNA

The mixture was incubated for 10 minutes at RT, 50 minutes at 37°C and 15 minutes at 70°C to inactivate the enzymes. The resulting cDNA was diluted either 1:5 or 1:10 and used for qPCR using the Invitrogen SYBR Green SuperMix. Reactions were run in 384-well plates on a BioRad C1000Touch / CFX384 Real-Time System as follows:

6.25 µl SYBR-Mix (2x)
3.25 µl H2O
0.25 µl of each primer
2.5 µl cDNA

The following primer pairs were used for qPCR amplification:

PolR2a (145 bp):	CTGGTCCTTCGAATCCGCATC
	GCTCGATACCCTGCAGGGTCA
NIK (164 bp):	CGAAACTGAGGACAACGAG
	CACACTGGAAGCCTGTCTG
Skint-1 (143 bp):	TTCAGATGGTCACAGCAAGC
	GAACCAGCGAATCTCCATGT
Aire (187 bp):	TGCATAGCATCCTGGACGGCTTCC
	CCTGGGCTGGAGACGCTCTTTGAG

BUFFERS

FACS buffer:

PBS (phosphate buffered saline, Kantonsapotheke Zurich)

2% FCS

0.005% Sodium Azide

MACS buffer:

PBS

5 g of bovine serum albumin (BSA, Sigma)

4 ml of 0.5M EDTA

Biopsy lysis buffer:

50 ml 1M Tris/HCl pH 7.5

5 ml 0.5M EDTA (AppliChem)

5 ml 20% SDS

20 ml 5M NaCl

ad 500ml with H_2O bidest

Red blood cell lysis buffer:

4.15 g NH_4Cl

0.55 g $KHCO_3$

0.185 g EDTA

ad 500 ml with H_2O bidest, sterile filtration

Homemade Perm/Wash Buffer:

PBS

2% BSA

0.5% Saponin

TAE buffer (50x):

484 g Tris base

14.2 ml acetic acid

200 ml 0.5M EDTA (pH 8.0)

adjust pH to 7.5

ad 2000 ml with H_2O dest

TE buffer:

10mM Tris/HCl pH 7.5

1mM EDTA (2 ml for 1000 ml of buffer)

Coating buffer:

0.2 M Sodium Phosphate, pH 6.5

12.49 g of Na_2HPO_4, 15.47 g NaH_2PO_4

ad 1000 ml with H_2O dest

Digestion medium:

RPMI (Gibco or PAN)

2% FCS

25mM HEPES

Cell culture medium for *in vitro* culture of lymphocytes:

IMDM (Gibco)

10% FCS

Penicillin/Streptomycin

L-Glutamine (final conc. 2mM)

β-Mercaptoethanol (final conc. 0.5mM)

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APPENDIX

The following manuscript has been accepted by the European Journal of Immunology (Eur. J. Immunol.) for publication and is currently in press.

THY1$^+$ SCA1$^+$ INNATE LYMPHOID CELLS INFILTRATE THE CNS DURING AUTOIMMUNE INFLAMMATION, BUT DO NOT CONTRIBUTE TO THE DISEASE DEVELOPMENT

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Key words: innate lymphoid cells (ILCs), depletion, autoimmunity, EAE, IL-23

Abbreviations used in this paper: ILC, innate lymphoid cell; EAE, experimental autoimmune encephalomyelitis; RORγt, retinoic receptor related orphan receptor; MOG, myelin oligodendrocyte glycoprotein; CNS, central nervous system; T_H17, T helper 17 cell; Lin, lineage;

SUMMARY

IL-23 is absolutely crucial for the development of T cell-driven autoimmune disease in mice. Even though IL-23 is widely held to be involved in the stabilization of IL-17-secreting T cells, naïve T cells lack the IL-23 receptor. Thus, the primary cellular target of IL-23 in the context of autoimmunity is a subject of some debate.
Innate lymphoid cells (ILCs) are a recently discovered family of lymphocytes being involved in early host defense, particularly at mucosal-epithelial surfaces. Given the fact that RORγt dependent ILCs (group 3 ILCs) constitutively express the IL-23-receptor, and that they have been implicated in intestinal autoimmunity, we hypothesized that ILCs could contribute to the early development of autoimmune neuro-inflammation. Through systematic analysis we detected a sizable population of $Thy1^+$ $Sca1^+$ ILCs in the inflamed CNS tissue. CNS-infiltrating ILCs were characterized by expression of the IL-7-receptor and production of pro-inflammatory IL-17 and IFN-γ. Furthermore, genetic fate-mapping revealed their dependence on the transcription factor RORγt. However, upon specific *in vivo* ablation of this cell population, we found that they do not influence the course of the disease.

INTRODUCTION

Over the past 5 years, the term innate lymphoid cells (ILCs) has been coined to describe a new family of innate lymphocytes that lack rearranged antigen receptors, but share phenotypic and functional characteristics with cells of the adaptive immune system. Beside the well-characterized populations of natural killer (NK) cells and lymphoid tissue inducer cells (LTIs), several subtypes of ILCs have recently been described, both in mouse and human (reviewed in (Spits and Cupedo, 2012) and (Spits and Di Santo, 2010)). $ROR\gamma t^+$ ILCs, which depend on the retinoic receptor related orphan receptor (RORγt) for their development, constitutively express the IL-23 receptor and are able to produce pro-inflammatory cytokines such as IL-17 and IL-22, similar to T cells of the T_H17 lineage (Takatori et al., 2009). In contrast, so-called group 2 ILCs (also known as nuocytes or natural helper cells) were discovered as innate producers of IL-5 and IL-13 (Moro et al., 2010; Neill et al., 2010). Very recently, a group of researchers has proposed a unifying nomenclature for ILCs, which would divide these cells into three subgroups based on their phenotypic and functional profile (Spits et al., 2013).
$ROR\gamma t^+$ ILCs (group 3 ILCs) are best known for their non-redundant role during formation of secondary lymphoid tissues in embryonic development (Eberl et al.,

2003), but they also have been suggested to be critical in early host defense in different mouse models of infection, in particular in the intestine. For example, after infection with *Citrobacter rodentium*, $CD4^+$ $Thy1^+$ ILCs respond by production of IL-22 required for bacterial clearance (Sonnenberg et al., 2011). Furthermore, $Nkp46^+$ ILCs have been implicated in the maintenance of intestinal homeostasis (Luci et al., 2009; Sawa et al., 2011).

In 2010, another unexpected role was attributed to $ROR\gamma t^+$ ILCs: Powrie and colleagues identified a $Lineage^-$ $Thy1^+$ $Sca1^+$ population of ILCs as the main mediator of innate IL-23-dependent gut inflammation in $Rag^{-/-}$ mice after infection with *Helicobacter hepaticus* (Buonocore et al., 2010). Of note, a follow-up report showed that ILCs are also enriched in intestinal biopsies from patients suffering from inflammatory bowel disease (IBD) or Crohns disease (Geremia et al., 2011). These unexpected findings suggest that ILCs play a critical role in autoimmune pathology. This hypothesis was corroborated by another study, in which lung natural helper cells, a population of Type 2 ILCs (group 2 ILCs), were shown to participate substantially in allergen-induced airway inflammation, at least in the murine system (Halim et al., 2012). Furthermore, it has been suggested that ILCs are able to influence adaptive immune responses in general via OX40 ligand signaling to memory T cells (Kim et al., 2005; 2003).

The development of autoimmune neuro-inflammation in the murine system is critically dependent on the cytokine IL-23 (Becher et al., 2002; Cua et al., 2003). Mice lacking the genes of IL-23, namely *Il23a* and *Il12b* or components of the IL-23 receptor complex are completely EAE resistant. However, even though IL-23 had initially been described to polarize IL-17 secreting auto-aggressive T cells (Langrish, 2005), it became later clear that other factors initiate the differentiation of T_H17 cells (Korn et al., 2009). In fact, naïve T cells are unresponsive to IL-23, as they lack the appropriate receptor complex (Zhou et al., 2007). Hence the actual function and cellular target of IL-23 in the context of neuro-inflammatory disease remains a subject of some debate. In contrast to naïve T helper cells, ILCs (as well as $\gamma\delta$ T cells) are constitutively responsive to IL-23 signaling and thus among the first cells sensing IL-23. Indeed, some reports suggested that the immediate IL-23 responsiveness of $\gamma\delta$ T cells can be a critical factor in models of autoimmune inflammation (Pantelyushin et al., 2012).

Thus, we hypothesized that ILCs could also play a role in initiating neuro-inflammation. So far, outside of lymphoid organs the presence of ILCs has only been investigated in the skin, lung and intestine (Spits and Cupedo, 2012). We analyzed the central nervous system (CNS) of mice immunized with the immunodominant peptide of the myelin oligodendrocyte glycoprotein (MOG_{35-55}) and indeed detected a

significant population of lineage negative Thy1$^+$ Sca1$^+$ ILCs, which were able to produce both IFN-γ and IL-17. A small population of these cells was also detectable in the CNS of naïve animals. Genetic fate-mapping revealed the major fraction of these cells belonging to the RORγt-dependent lineage (group 3 ILCs), but a minor fraction of CNS-infiltrating ILCs resembled a Thy1$^+$ RORγt-independent lineage (group 2 ILCs). However, *in vivo* ablation of all Thy1$^+$ ILCs demonstrated that these cells did not contribute significantly to disease progression, indicating that their presence in the CNS is a result of the inflammation dictated by adaptive immunity and that their contribution to the inflammatory process is negligible.

RESULTS

THY1$^+$ SCA1$^+$ ILCS INFILTRATE THE CNS DURING NEURO-INFLAMMATION

Phenotypically, the ILC family has been characterized by a large variety of markers, which led to a plethora of subtypes and designations for ILCs (Spits and Cupedo, 2012). Only recently efforts have been undertaken to put forward a uniform nomenclature for ILCs (Spits et al., 2013; Walker et al., 2013). Expression of markers such as Nkp46, CD117 (c-kit) or CD4 has been reported only in certain experimental settings (Buonocore et al., 2010; Eisenring et al., 2010; Spits and Cupedo, 2012; Spits et al., 2013). When looking for accordance in the public domain, besides being Lineage (lin) negative, all reported subtypes of ILCs express IL-7Rα (CD127) – in line with their dependence on common gamma chain cytokines for development (Meier et al., 2007) – and Thy1. Thus, for our analysis of ILCs during CNS autoimmunity, we focused on the above-mentioned markers as being essential for their identification.

When analyzing the CNS of EAE-diseased wildtype mice by multicolor flow cytometry, we used separate fluorescent channels to firmly exclude lin$^+$ cells, particularly T cells. Of note, in many published reports lin$^+$ cells were excluded by use of a single dump channel (Geremia et al., 2011; Monticelli et al., 2011), ignoring the fact that different lineage markers show a high variability in their staining brightness. By analyzing the CNS-infiltrating lymphocyte fraction, gating on CD45$^+$ CD11b$^-$ B220$^-$ CD3$^-$ CD5$^-$ cells revealed a considerable population of Thy1$^+$ Sca1$^+$ ILCs expressing IL-7Rα (Fig. 1a). These cells stained negative both for CD4 and Nkp46 (Fig. 1b), which is in line with the phenotype attributed to ILCs in intestinal autoimmune inflammation (Buonocore et al., 2010). Expression of c-kit (CD117) was also not detectable, and only a minor fraction of Thy1$^+$ Sca1$^+$ ILCs expressed Nk1-1.

In addition to Thy1$^+$ Sca1$^+$ ILCs a population of Thy1$^+$ Sca1$^-$ cells was also consistently present in the inflamed CNS. Phenotypic analysis of these cells revealed that they did not express the IL-7Rα, but instead NK1.1 and Nkp46 (Fig. 1b), suggesting that these cells belong to the NK cell lineage, which have been categorized also as group 1 ILCs. Indeed, some NK cells have been reported to express Thy1, consistent with our analysis (Kupz et al., 2013).

To analyze whether CNS-infiltrating ILCs were of the RORγt-dependent lineage, we took advantage of a RORc-fatemapping system: Mice expressing Cre-recombinase under control of the RORc promotor were crossed to R26-YFP^{STOPflox} animals. In the resulting RORc-YFP mice all cells that once expressed RORγt during their development are terminally marked with YFP (Vonarbourg et al., 2010). Indeed, the majority of Thy1$^+$ Sca1$^+$ ILCs in the inflamed CNS was positive for YFP (Fig. 1c), while a minor fraction of the infiltrating cells seemed to derive from a RORγt-independend lineage, phenotypically resembling group 2 ILCs. The majority of Thy1$^+$ Sca1$^-$ cells showed no YFP signal, which is in line with their categorization as NK cells (Fig 1c).

In order to evaluate whether the CNS infiltrating ILCs still express RORγt, we used a RORc-GFP reporter strain (Eberl et al., 2003). Interestingly, we found that in the inflamed CNS of these animals, only a minority of Thy1$^+$ Sca1$^+$ ILCs retained RORγt expression. This is in line with published work by Diefenbach and colleagues showing that a sizable fraction of RORγt-dependent ILCs lose RORγt expression during their differentiation or activation (Vonarbourg et al., 2010).

Given the unexpected finding that ILCs can be detected in the CNS during peptide-induced autoimmune inflammation, we wanted to clarify whether these cells are also present under steady state conditions. Using the same gating strategy as in Fig. 1a, a small population of Lin$^-$ Thy1$^+$ Sca1$^+$ ILCs could consistently be detected in healthy wildtype animals (Fig. 1d). To exclude artifacts resulting from a potential inadvertent inclusion of T cells, we also analyzed Rag1$^{-/-}$ mice, which completely lack T and B cells, as well as TCR$\beta\delta^{-/-}$ mice, which lack all T cells. Indeed, we could verify that the CNS of healthy Rag1$^{-/-}$ as well as TCR$\beta\delta^{-/-}$ mice also contained a population of Lin$^-$ Thy1$^+$ Sca1$^+$ cells. IL-7Rα expression was detectable irrespective of the analyzed genotype (Fig. 1d).

Quantification showed that the amount of ILCs in the CNS during steady state conditions, both in absolute numbers as well as in percentage, was similar in WT, Rag$^{-/-}$ and TCR$\beta\delta^{-/-}$ animals (Fig. 1e). Due to their lack of lineage markers and their rarity, their precise location within the un-inflamed CNS is thus far unclear.

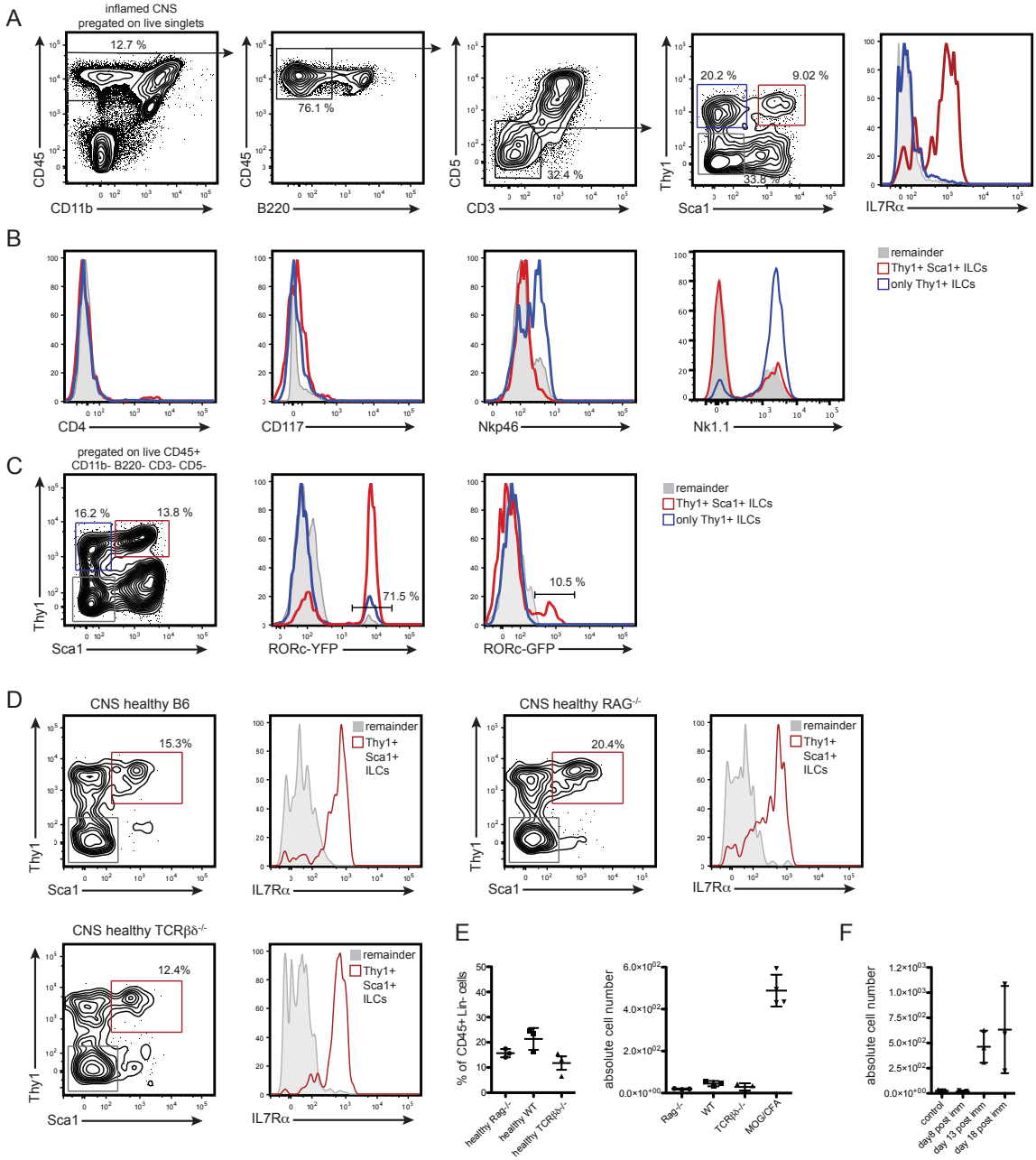

Figure 1. **Lineage$^-$ Thy1$^+$ Sca1$^+$ ILCs invade the central nervous system during neuro-inflammation.**
(A) Lymphocytes were isolated from MOG/CFA-immunized WT or heterozygous RORc-GFP reporter mice showing an EAE score of at least 2.0 and analyzed by flow cytometry. After exclusion of duplets and dead cells, CD45$^+$ lymphocytes were gated for lineage negative (CD11b$^-$ B220$^-$ CD3$^-$ CD5$^-$) cells. Upper right panels depict the presence of Thy1$^+$ Sca1$^+$ ILCs and Thy1$^+$ Sca1$^-$ cells and expression of IL-7Rα (CD127). Data are representative of at least three independent experiments, plots show pooled data from three individual CNS samples.
(B) Further phenotypic analysis of CNS infiltrating Thy1$^+$ Sca1$^+$ ILCs (depicted in red) shows no expression of neither CD4 nor Nkp46. Blue histograms show Thy1$^+$ Sca1$^-$ cells, and shaded histograms show remaining Thy1$^-$ Sca$^-$1 cells.
(C) CNS infiltrating cells were isolated from MOG-immunized RORc-YFP fate-map (left plot) or RORc-GFP reporter mice (right plot) and analyzed for YFP and GFP expression, respectively. Thy1$^+$ Sca1$^+$ ILCs are depicted in red, Thy1$^+$ Sca1$^-$ cells are depicted in blue.

(D) Lymphocytes were isolated from the steady-state CNS of wildtype, Rag1$^{-/-}$ and TCR$\beta\delta^{-/-}$ mice. Using the same gating strategy as in (A), a comparable population of Thy1^{+} Sca1^{+} ILCs was detected in all three genotypes. Histograms depict IL-7Rα expression on Thy1^{+} Sca1^{+} ILCs (red). Data are representative of at least two individual experiments, plots show pooled data of three individual CNS samples.
(E) Quantification of brain-infiltrating Thy1^{+} Sca1^{+} ILCs in percentage of CD45^{+} Lin^{-} cells (left panel) as well as absolute cell numbers (right panel). Plots depict individual animals and mean +/- SD.
(F) Quantification of brain-infiltrating Thy1^{+} Sca1^{+} ILCs at different timepoints after immunization in comparison to healthy controls. Plots depict individual animals and mean +/- SD.

In contrast to the steady state, a drastic increase in ILCs was observed under inflammatory conditions (Fig. 1e), suggesting that Thy1^{+} Sca1^{+} ILCs infiltrate into or expand in the CNS during experimental autoimmunity. In order to obtain a more detailed view on the temporal expansion of ILCs we analyzed the CNS of MOG/CFA immunized animals at different time-points post immunization, namely on day 8 (prior to disease onset), day 13 (peak disease) and day 18 (post peak disease). While prior to disease onset very few Thy1^{+} Sca1^{+} ILCs could be detected, the number of ILCs on day 13 and day 18 post immunization was comparable. However, ILCs numbers vary at later disease time points, potentially correlating with the extent of remission from the disease.

CNS-INVADING ILCS CONTRIBUTE TO LOCAL CYTOKINE PRODUCTION

One of the most prominently studied features of RORγt^{+} ILCs is their immediate responsiveness to IL-23 and their ability to produce pro-inflammatory cytokines, including IL-17 (Takatori et al., 2009), IL-22 (Luci et al., 2009) but also IFN-γ (Buonocore et al., 2010). In innate intestinal inflammation, both IL-17 and IFN-γ produced by ILCs have been shown to greatly contribute to
disease progression(Buonocore et al., 2010). Therefore, we analyzed cytokine production of CNS-infiltrating ILCs *ex vivo* by intracellular cytokine staining and found that a large population of Thy1^{+} Sca1^{+} ILCs was able to produce IFN-γ, and to a lesser extent IL-17 (Fig. 2a). We could not detect any expression of IL-22 (data not shown). Analysis of cytokine expression by CNS-resident ILCs during steady state showed only minor production of both IFN-γ and IL-17 (Fig. 2b).
Since PMA/Ionomycin is a very strong activator, we asked whether cytokine production by Thy1^{+} Sca1^{+} ILCs could be directly induced by stimulation with IL-23. Indeed, *in vitro* culture in the presence of IL-23 induced IL-17 production by CNS isolated ILCs comparable to the levels observed with PMA/Ionocycin (Fig 2c).

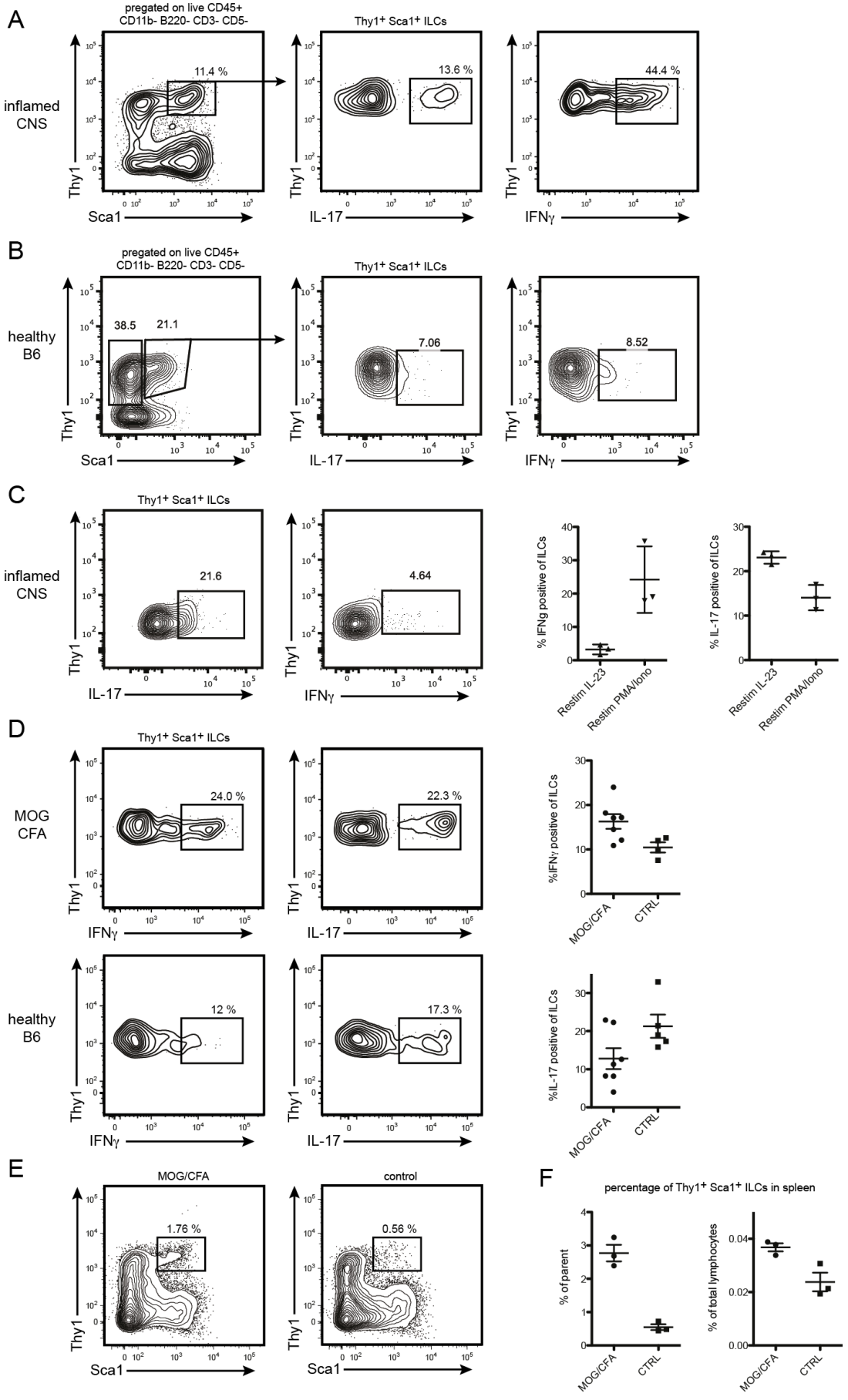
A
pregated on live CD45+
CD11b- B220- CD3- CD5-
Thy1+ Sca1+ ILCs
inflamed CNS
11.4 %
13.6 %
44.4 %
Thy1
Sca1
IL-17
IFNγ
B
pregated on live CD45+
CD11b- B220- CD3- CD5-
Thy1+ Sca1+ ILCs
healthy B6
38.5
21.1
7.06
8.52
C
Thy1+ Sca1+ ILCs
inflamed CNS
21.6
4.64
% IFNg positive of ILCs
% IL-17 positive of ILCs
Restim IL-23
Restim PMA/Iono
D
Thy1+ Sca1+ ILCs
MOG CFA
24.0 %
22.3 %
%IFNγ positive of ILCs
%IL-17 positive of ILCs
MOG/CFA
CTRL
healthy B6
12 %
17.3 %
E
MOG/CFA
control
1.76 %
0.56 %
F
percentage of Thy1+ Sca1+ ILCs in spleen
% of parent
% of total lymphocytes

Figure 2: **CNS infiltrating ILCs produce proinflammatory cytokines.**
(A) Lymphocytes were isolated from MOG/CFA-immunized wildtype mice showing an EAE score of at least 2.0 and restimulated *in vitro* with PMA/Ionomycin. Using the same linage gating strategy as in Fig. 1a, $Thy1^+$ $Sca1^+$ ILCs were analyzed for production of IFN-γ (right panel) and IL-17 (middle panel). Plots are representative of at least three independent experiments and depict pooled data of three individual CNS samples.
(B) $Thy1^+$ $Sca1^+$ ILCs isolated from the steady-state CNS of healthy B6 animals were restimulated *in vitro* with PMA/Ionomycin/Brefeldin A and analyzed for production of IFN-γ and IL-17. Plots represent pooled data from three individual animals.
(C) $Thy1^+$ $Sca1^+$ ILCs from MOG/CFA immunized wildtype mice were restimulated *in vitro* with IL-23/Brefeldin A and assessed for production of IFN-γ and IL-17. Flow plots show representative data out of two experiments.
(D) Lymphocytes were obtained from the spleen of MOG/CFA-immunized wildtype mice after onset of clinical EAE symptoms (upper panel) as well as healthy controls (lower panel) and stained for $Lineage^-$ $Thy1^+$ $Sca1^+$ ILCs and IFN-γ and IL-17. Quantification of cytokine production is summarized in the two right plots, showing individual animals and mean +/- SD.
(E) Representative plot of Lineage negative $Thy1^+$ $Sca1^+$ ILCs in the spleen of MOG/CFA immunized wildtype mice after onset of EAE symptoms and healthy controls. F: Quantification of $Thy1^+$ $Sca1^+$ ILCs among total lymphocytes (right plot) and as percent of parent Lin^- cells (left plot). Plots show individual animals and mean +/- SD.

However, very little IFN-γ expression was induced by IL-23, consistent with what is known about the impact of IL-23 on ILCs.

It has already been demonstrated that peripheral ILCs can react very rapidly (within hours) to danger signals such as Zymosan (Takatori et al., 2009) or also indirectly to TLR-5 stimulation (Van Maele et al., 2010). We thus wanted to determine whether the immunization with CFA would trigger systemic ILC expansion in draining lymph nodes and thus allow their accumulation within the inflamed CNS. In the peripheral immune compartment, we failed to detect any significant difference between ILCs in healthy control and MOG/CFA treated animals in terms of cytokine production, although there was a slight trend towards increased levels of IL-17 and reduced levels of IFN-γ (Fig. 2d). However, after MOG/CFA immunization the total percentage of ILCs in the splenic lymphocyte pool increased approximately two to fourfold (Fig. 2e).

IN VIVO ABLATION OF $THY1^+$ ILCS REVEALS THEIR REDUNDANCE FOR NEURO-INFLAMMATION

The presence or absence of a cell type in an inflamed organ does not necessarily correspond with an important role during disease progression. Such correlative observations have often led to erroneous assumptions regarding causal relationships. Thus, we decided to systematically test whether the increased number of ILCs in the CNS during inflammation has any impact on disease progression or severity.

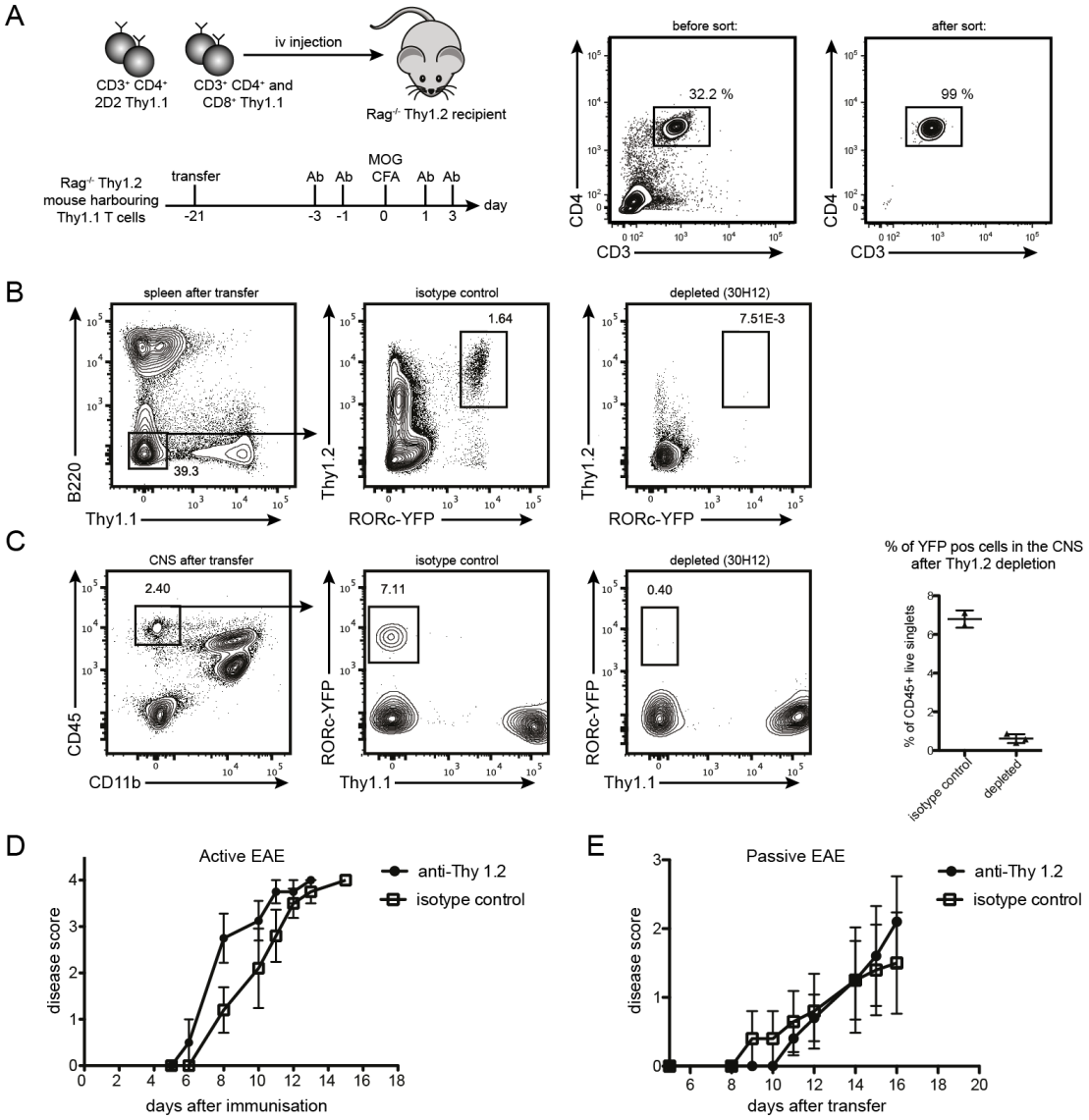

Figure 3: **Depletion of Thy1$^+$ ILCs does not influence disease progression of EAE.**
(A) Schematic overview of the depletion experiments. Peripheral CD3$^+$ CD4$^+$ T cells from a 2D2 Thy1.1 donor as well as CD3$^+$CD4$^+$ and CD3$^+$ CD8$^+$ T cells from a Thy1.1 donor were sorted, and 2x10^6 total cells were intravenously transferred to Rag1$^{-/-}$ or RORc-YFP Rag1$^{-/-}$ recipients. After allowing for homeostatic expansion of the transferred T cells, mice were treated either with depleting anti-Thy1.2 antibody (clone 30H12) or with isotype control at the indicated timepoints ("Ab"), followed by immunization with MOG/CFA. Right panels depict the purity of the sorted T cells and shows representative data of more than three individual experiments.
(B and C) Efficient depletion of both Thy1$^+$ and Thy1$^+$ YFP$^+$ cells in the spleen (B) and CNS (C) was assessed in parallel RORc-YFP Rag1$^{-/-}$ animals after four injections of 200 µg of anti-Thy1.2 (clone 30H12). For flow cytometric analysis, Thy1.2 was stained using a different antibody clone (53.2-1). Representative plots for two individual experiments are shown. Right panel shows individual animals, mean +/- SD. (D and E) Disease score after active MOG/CFA immunization
(D) and after passive transfer EAE of *in vitro* restimulated Thy1.1 T cells into isotype control or 30H12-treated Rag1$^{-/-}$ recipients (E). Data are representative of at least three independent experiments. Disease scores are shown as mean +/- SEM.

To do so, we devised an experimental system that allows selective depletion of all Thy1$^+$ ILCs (targeting both group 2 and group 3 ILCs, irrespective of their dependence on RORγt) during active immunization, without affecting T cells that also express Thy1. CD4$^+$ T cells obtained from TCR-transgenic 2D2 animals (specific for the MOG-peptide, (Bettelli et al., 2003)) bred to a Thy1.1 background and CD4$^+$ as well as CD8$^+$ T cells obtained from wildtype Thy1.1 animals were sorted to high purity (Fig. 3a). A mixture of these T cells was transferred to Rag1$^{-/-}$ recipients with a Thy1.2 background. Hence, the endogenous population of ILCs would express the Thy1.2 marker. After three weeks, to allow for homeostatic expansion of the transferred Thy1.1$^+$ T-cell populations, depletion of host-derived ILCs was started using an anti Thy1.2 antibody (clone 30H12). The experimental layout is schematically summarized in Fig. 3a.

In parallel to the actual immunization experiments, we assessed depletion of Thy1$^+$ cells after four injections of 200 µg of anti-Thy1.2. To do so, we used a different clone of Thy1.2 for staining (53.2-1) than for depletion (30H12), showing that all Thy1$^+$ cells in the spleen were depleted with this protocol (Fig. 3b).

Furthermore, in this experimental setting we also used RORc-YFP mice bred on a Rag$^{-/-}$ background as recipients for the T-cell transfer. In this case, RORγt - dependent Thy1$^+$ ILCs can be tracked by their expression of YFP. Analysis of spleen and also CNS after four injections of anti-Thy1.2 showed that the majority of YFP$^+$ cells were depleted in either organ, suggesting that our depletion protocol efficiently targets Thy1$^+$ YFP$^+$ ILCs also in the CNS (Fig. 3c).

After immunization with MOG/CFA, comparison of disease scores between isotype-treated control animals and ILC depleted animals revealed no difference in disease severity and incidence, suggesting that ILCs are superfluous during CNS autoimmunity, at least in the EAE model (Fig. 3d). To substantiate this finding, we performed passive EAE transfer experiments of *in vivo* primed Thy1.1 T cells into Thy1.2-depleted Rag1$^{-/-}$ recipients, where we also could not detect any differences in disease progression after ILC-depletion. (Fig. 3e).

In summary, our data suggest that during autoimmune neuro-inflammation, Thy1+ ILCs do not play a critical role in disease development or progression.

DISCUSSION

During the last decade it became obvious that one of the most critical factors in many autoimmune pathologies is IL-23. Particularly in neuro-inflammation, IL-23 has turned out to be a non-redundant factor, but the mechanism underlying its action is far from being understood. IL-23 can trigger differentiation of αβ T cells towards IL-17 T_H17 cells (Langrish, 2005) and GM-CSF producing T cells (Codarri et al., 2011), but naïve T cells do not express the IL-23 receptor. In contrast, ILCs as s well as γδ T cells have been shown to constitutively express IL-23R, and in the case of γδ T cells a significant contribution to the pathogenesis of EAE (Petermann et al., 2010) as well as psoriatic skin inflammation has been reported (Cai et al., 2011; Pantelyushin et al., 2012). Furthermore, the recent finding that intestinal ILCs via expression of MHC class II are able to regulate CD4 T-cell responses (Hepworth et al., 2013) further emphasizes their so far underestimated role in the adult immune system.

Along these lines, we hypothesized that ILCs, via their immediate responsiveness to IL-23 signals, contribute to autoimmune neuro-inflammation. Further support for this hypothesis came from the fact that ILCs are critical players in IL-23 driven innate gut inflammation (Buonocore et al., 2010). Indeed, we could show that ILCs are not only present at mucosal surfaces as previously reported, but also in the CNS both during steady state and inflammation. Based on their surface marker profile, the majority of CNS-infiltrating ILCs resembled what had been categorized as RORγt-dependent, IL-17 producing group 3 ILCs (Spits and Cupedo, 2012; Spits et al., 2013), with only a minor fraction resembling group 2 ILCs. However, the lineage releationships within the ILC family are only starting to be unraveled (Sawa et al., 2010; Vonarbourg et al., 2010; Walker et al., 2013), and what is now considered to be a separate lineage might indeed only represent a different activation state.

Interestingly, under inflammatory conditions the majority of CNS-infiltrating ILCs ceased to express RORγt, in line with published work suggesting that during their differentiation certain ILC populations lose RORγt expression (Vonarbourg et al., 2010). Of note, in this autoimmune colitis model the RORγt and CD4 negative ILC population was causative for gut pathology (Vonarbourg et al., 2010). Of note, it has also been proposed that expression of T-bet in RORγt$^+$ ILCs can further modulate their fate and function, causing a switch from a homeostatic to a pro-inflammatory phenotype (Klose et al., 2013).

Given their presence as a substantial, cytokine-producing population in the CNS, we were surprised to discover that depletion of Thy1$^+$ ILCs did not alter progression of EAE. There are several possible explanations: First, the widely used immunization

protocol utilizing MOG/CFA for induction of EAE might be an inappropriate trigger for ILCs. Second, the overwhelming amount of activated, MOG-reactive T cells might mask a possibly subtle role of ILCs during the course of autoimmunity. Third, ILCs do not play an important role in this particular setting of autoimmune inflammation.

In summary, we identified a CNS-invading population of group 3 ILCs with the capacity to secrete cytokines locally. However, using a functional depletion model targeting all $Thy1^+$ ILC subsets we have thoroughly ruled out the involvement of ILCs in the pathogenesis of EAE. Nevertheless, since the initial trigger for human MS is still unknown, it cannot be excluded that ILCs participate in this primary event. Lastly, even though the precise function and cellular targets of IL-23 remain elusive, we can herewith exclude a vital role of ILCs as pathologically relevant responders to IL-23 during autoimmune neuro-inflammmation.

MATERIAL AND METHODS

MICE AND ANIMAL EXPERIMENTS

C57BL/6 (wildtype), congenic C57BL/6 Thy1.1, *Rag1*$^{-/-}$, *TCR*$\beta\delta^{-/-}$ mice as well as *Rorc-GFP* mice were purchased from Jackson Laboratories and bred in-house under specific pathogen-free (SPF) conditions. *Rorc-GFP* mice were only used as heterozygous reporter animals. *Rorc-Cre* and *R26-YFP*^{STOPflox} mice were obtained from Andreas Diefenbach and bred in-house either on a WT or a Rag$^{-/-}$ background. EAE was induced as described previously (Gyülvészi et al., 2009). Briefly, mice were immunized subcutaneously with 200 ug of MOG_{35-55} peptide (MEVGWYRSPFS-RVVHLYRNGK; GenScript) emulsified in CFA (Difco) and two intraperitoneal injections of 200 ng Pertussis toxin (Sigma) on day 0 and 2. For passive EAE experiments, spleen and lymph node cells were harvested from C57BL/6 Thy1.1 donor mice on day 7 after immunisation, restimulated two days with 20ug/ml MOG and 10 ng/ml IL-23 and then i.v. transferred to *Rag1-/-* recipients.

All animal experiments were approved by local authorities (Swiss veterinary office, canton Zurich, licence 55/2009 and 85/2012). Depleting antibodies used in some experiments (rat-anti-mouse-Thy1.2, clone 30H12 and isotype control ratIgG2b, clone LTF-2) were obtained from BioXCell (West Lebanon, USA).

LYMPHOCYTE ISOLATION FROM CNS

For peak disease analysis, animals were euthanasized on day 13-16 post immunization. Mononucleated cells were obtained from CNS tissues as described (Gyülvészi et al., 2009): mice were euthanized using CO_2 inhalation. Afterwards, animals were perfused using ice-cold PBS and brain and spinal cord were collected. Tissues were cut into small pieces using scissors, followed by 30 minutes of digestion with 0.4 mgl/ml Collagenase D (Roche) and 0.5 mg/ml DNase (Sigma) in IMDM containing 25mM HEPES and 2% FCS. Remaining pieces of tissue were homogenized using syringes and 20 gauge needles. After washing, the cell suspension was loaded onto a continuous 28% Percoll (GE) gradient and centrifuged for 30 minutes at 15.000 g. Mononuclear cells were obtained from the interphase, washed twice with PBS and used for further procedures.

FLOW CYTOMETRY

Flow cytometric analysis was performed following standard methods (reviewed in (Perfetto et al., 2004)). The flourochrome-conjugated antibodies used were obtained

either from BD, BioLegend or eBioscience. In all stainings, dead cells were excluded using an Aqua Live/Dead fixable staining reagent (Invitrogen), and duplets were excluded by FSC-A vs FSC-H gating. For intracellular cytokine staining, cells were incubated 4 hours in IMDM containing 10% FCS with PMA (50ng/ml) / Ionomycin (500ng/ml) and GolgiPlug (Brefeldin A, BD). For some experiments, cells were restimulated for 4 hours in the presence of IL23-Fc (generated in the lab) and GolgiPlug (BD). Cytofix/Cytoperm (BD) was used according to the manufacturers instructions. Analysis was performed using a LSR II Fortessa (special order research product, BD and equipped with 405 nm, 488 nm, 561 nm and 640 nm laser lines), cell sorting was carried out using a FACSAria III (BD). Data analysis was done using FlowJo V9.x and 10.0.0.x (Treestar). For some plots, data from several individual samples were concatenated (pooled) in FlowJo.

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EDUCATION

12-2008 – present:	PhD studies in the lab of Prof. Burkhard Becher, Institute of Experimental Immunology, University of Zurich, Switzerland Thesis title: "The NFkB inducing kinase – Regulating the Function of Dendritic Cells and γδ T cells" Member of the PhD program "Molecular Life Sciences", Life Science Zurich Graduate School
07-2008:	Completion of the studies "Molecular Biology", second part of studies and final exams passed with distinction. Academic degree: Magister rer. nat. (equivalent to MSc)
03-2007 – 05-2008:	Diploma thesis in the lab of Prof. Ludger Klein, Institute of Molecular Pathology (IMP), Vienna, Austria. Thesis title: "Delineating a phenotypic and functional roadmap to regulatory T cell development at the $CD4^+$ single positive stage"
10-2002 – 07-2008:	Studies of „Molecular Biology" at the University of Vienna, specialisation in immunology, cell biology and structural biology
06-2002:	Graduation from grammar school (Innsbruck, Austria), passed with distinction

FURTHER ACTIVITIES

03-2013:	Co-initiator and organizer of the first comprehensive course on flow cytometry at the UZH, organized for the Life Science Zurich graduate school
01-2012 – present:	Co-organiser of a monthly seminar series on flow cytometry
02-2011 – present:	Sorting operator and user support at the Flow Cytometry Facility (Head: Claudia Dumrese) of the University of Zurich, Switzerland.
12-2009 – present:	Various teaching activities for the Institute of Experimental Immunology, including supervision of master students
05-2005 – 12-2005:	Corrector of the book "Coffee House notes on Virology" (written by Prof. Timothy Skern, University of Vienna)

SCHOLARSHIPS AND AWARDS

05-2011: Poster award at the 6th ENII immunology summer school, Title: "Loss of NIK signalling in dendritic cells but not in T cells abrogates T effector function during autoimmunity

06-2010: Junior fellowship ("Forschungskredit") from the University of Zurich, Title: "The NFκB-inducing kinase (NIK) – role and function during autoimmunity"

08-2009: Poster award at the retreat of the Molecular Life Science PhD Program, Title: "The NFκB-inducing kinase (NIK) – extending our view on autoimmunity and inflammation"

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LANGUAGE AND ADDITIONAL SKILLS

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Computer skills: profound knowledge in Microsoft Office, Adobe Indesign, Adobe Illustrator, Adobe Photoshop, GraphPad Prism

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Non-academic qualifications: Certified Mountain Bike guide (DIMB)

CONFERENCES AND MEETINGS ATTENDED

2nd NIF (Network of Immunology Frontiers) winter school on advanced Immunology, Singapore, January 2013 (oral and poster presentation)

11th International Congress of Neuroimmunology and 12th ESNI course on Neuroimmunology, Boston, USA, November 2012 (poster presentation)

27th Congress of the International Society for Advancement of Cytometry (CYTO), Leipzig, Germany, June 2012

6th ENII Immunology summer school, Sardinia, Italy, May 2011 (oral and poster presentation)

22nd & 23rd Wolfsberg Meeting of Swiss Immunology PhD students, Switzerland, spring 2010 and 2011 (oral presentation)

3rd World Immune Regulation Meeting, Davos, Switzerland, March 2009

PUBLICATIONS

A. Sledzińska, S. Hemmers, *F. Mair*, O. Gorka, J. Ruland, L. Fairbairn, A. Nissler, W. Müller, A. Waisman, B. Becher, T. Buch: "TGF-β signaling is required for $CD4^+$ T cell homeostasis but dispensable for regulatory T cell function", PLoS Biol., in press

F. Mair and B. Becher: "Thy1$^+$ Sca1$^+$ innate lymphoid cells infiltrate the CNS during autoimmune inflammation, but do not contribute to the disease development", Eur J Immunol, in press

J. vom Berg, S. Prokop, K. Miller, J. Obst, R. Kälin, I. Lopategui-Cabezas, A. Wegner, *F. Mair*, C. G Schipke, O. Peters, Y. Winter, B. Becher & F. L Heppner: "Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease–like pathology and cognitive decline", Nat Med. 2012 Dec;18(12):1812-9.

AL. Croxford*, *F. Mair**, B. Becher: "IL-23: one cytokine in control of autoimmunity", Eur J Immunol. 2012 Sep;42(9):2263-73. (*equal contribution)

J. Hofmann*, *F. Mair**, M. Greter, M. Schmidt-Supprian, B. Becher: "NIK signaling in dendritic cells but not in T cells is required for the development of effector T cells and cell-mediated immune responses", J. Exp. Med. 2011 Aug 1, 208: 1917–1929. (*equal contribution)

G. Wirnsberger*, *F. Mair**, L. Klein: "Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control", PNAS 2009 Jun 23, 106(25): 10278-83 (*equal contribution)